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(57) Abstract

The present invention relates to fragments, especially variable fragments of immunoglobulins which are by nature devoid of light chains, these fragments being nevertheless capable of exhibiting a recognition and binding activity toward specific antigens. The present invention further relates to the use of such immunoglobulir fragments formed of at least one heavy chain variable fragment or derived therefrom, for therapeutic or veterinary purposes and especially for passive immunotherapy or serotherapy.

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Variable fragments of immunoglobulins - Use for therapeutic or veterinary purposes

The present invention relates to fragments, especially variable fragments of immunoglobulins which are by nature devoid of light chains these fragments being nevertheless capable of exhibiting a recognition and binding activity toward specific antigens. These fragments of immunoglobulins can for example be obtained by the expression in host cells for example in prokaryotic cells or eukaryotic cells of nucleotide sequences obtained from animals naturally expressing so-called "two-chain immunoglobulins", for instance from animals of the camelid family.

The present invention further relates to the use of such immunoglobulin fragments formed of at least one heavy chain variable fragment or derived therefrom, for therapeutic or veterinary purposes and especially for passive immunotherapy or serotherapy.

Functional immunoglobulins devoid of light polypeptide chains termed «two-chain immunoglobulin » or « heavy-chain immunoglobulin » have been obtained from animals of the family of camelids and have been described in an international patent application published under number WO 94/04678, together with two publications, especially Hamers-Casterman et al, 1993 and Muyldermans et al, 1994).

The isolation and characterization of these immunoglobulins, together with their cloning and sequencing have been described in the above referrenced documents which are incorporated by reference in the present application.

According to WO 94/04678 it has been established that different molecules can be isolated from animals which naturally produce them, which molecules have functional properties of the well known four-chain immunoglobulins these functions being in some cases related to

structural elements which are distinct from those involved in the function of four-chain immunoglobulins due for instance to the absence of light chains.

These immunoglobulins having only two chains, neither correspond to fragments obtained for instance by the degradation in particular the enzymatic degradation of a natural four-chain model immunoglobulin, nor correspond to the expression in host cells, of DNA coding for the constant or the variable regions of a natural four-chain model immunoglobulin or a part of these regions, nor correspond to antibodies produced in lymphopaties for example in mice, rats or human.

The immunoglobulins devoid of light chains are such that the variable domains of their heavy chains have properties differing from those of the four-chain immunoglobulin variable heavy chain (V_H). For clarity reasons, this variable domain according to the invention will be called V_{HH} in this text to distinguish it from the classical V_H of four-chain immunoglobulins.The variable domain of a heavy-chain immunoglobulin according to the invention has no normal interaction sites with the V_L or with the C_H1 domain which do not exist in the heavy-chain immunoglobulins. It is hence a novel fragment in many of its properties such as solubility and conformation of main chains. Indeed the V_{HH} of the invention can adopt a three-dimensional organization which distinguishes from the three-dimensional organization of known four-chain immunoglobulins according to the description which is given by Chothier C. and Lesk A.M, (1987- J.Mol. Biol. 197, 901-917).

According to the results presented in patent application WO 94/04678, the antigen binding sites of the isolated immunoglobulins, naturally devoid of light chains are located on the variable region of their heavy chains. In most cases, each heavy chain variable region of these two-chain immunoglobulins can comprise an antigen binding site.

A further characteristic of these two-chain immunoglobulins is that their heavy polypeptide chains contain a variable region (V_{HH}) and a

constant region (C_H) according to the definition of Roitt et al but are devoid of the first domain of the constant region is called C_H1.

These immunoglobulins of the type described hereabove can comprise type G immunoglobulins and especially immunoglobulins which are termed immunoglobulins of class 2 (IgG2) or immunoglobulins of class 3 (IgG3), according to the classification established in patent application WO 94/04678 or in the publication of Muyldermans et al (Protein Engineering Vol.7, N°9, pp 1129-1135-1994).

The absence of the light chain and of the first constant domain lead to a modification of the nomenclature of the immunoglobulin fragments obtained by enzymatic digestion, according to Roitt et al.

The terms Fc and pFc on the one hand, Fc' and pFc' on the other hand corresponding respectively to the papain and pepsin digestion fragments are maintained.

The terms Fab, $F(ab)_2$,$F(ab')_2$,Fabc, Fd and fv are no longer applicable in their original sense as these fragments have either a light chain, the variable part of the light chain or the CH_1 domain.

The fragments obtained by papain digestion or by V8 digestion, composed of the V_{HH} domain of the hinge region will be called $FV_{HH}h$ or $F(V_{HH}h)2$ depending upon whether or not they remain linked by the disulphide bonds.

The immunoglobulins referring to the hereabove given definitions can be originating from animals especially from animals of the camelid family. These heavy-chain immunoglobulins which are present in camelids are not associated with a pathological situation which would induce the production of abnormal antibodies with respect to the four-chain immunoblobulins. On the basis of a comparative study of old world camelids (Camelus bactrianus and Camelus dromaderius) and new world camelids (for example Lama Paccos, Lama Glama, and Lama Vicugna) the inventors have shown that the immunoglobulins devoid of light polypeptide chains are found in all species. Nevertheless differences may be apparent

in molecular weight of these immunobglobulins depending on the animals. Especially the molecular weight of a heavy chain contained in these immunoglobulins can be from approximately 43 kd to approximately 47 kd, in particular 45 kd.

Advantageously the heavy-chain immunoglobulins of the invention are secreted in blood of camelids.

The variable fragments of heavy chains of Immunoglobulins devoid of light chains can be prepared starting from immunoglobulins obtainable by purification from serum of camelids according to the process for the purification as described in detail in the examples of WO 94/04678. The variable fragments can also be obtained from heavy-chain immunoglobulins by digestion with papain or V8 enzymes.

These fragments can also be generated in host cells by genetic engineering or by chemical synthesis. Appropriate host cells are for instance bacteria (e.g. E. coli) eucaryotic cells including yeasts or animal cells including mammalian cells, or plant cells.

The observation by the inventors that Camelidae produce a substantial proportion of their functional immunoglobulins as a homodimer of heavy chains lacking the C_H1 domain and devoid of light chains(Hamers-Casterman et al, 1993), led to the proposal of having recourse to an immunized camel to generate and select single variable antibody fragments (V_{HH}) and furthermore give access to the corresponding nucleotide sequences.

Cloned camel single V_{HH} fragments were displayed on bacteriophages for selection and in bacteria for the large scale production of the soluble proteins, and were shown to possess a superior solubility behaviour and affinity properties compared to the mouse or human V_H equivalents (Muyldermans et al, 1994). Following this strategy, one would obtain small ligand binding molecules (MW around 16,000 D) which are not hindered by the presence of an oligopeptide linker (Borrebaeck *et al.*, 1992) or not inactivated by the disassembly of the VH-VL complex

(Glockshuber *et al.*, 1990). The camel V_{HH} fragments have the additional advantage that they are characteristic of the heavy chain antibodies which are matured in vivo in the absence of light chains.

The inventors have obtained evidence that variable fragments of high chains of immunoglobulins devoid of light chains can display an effective therapeutic activity when they are generated against a determined antigen.

To develop this technology of preparing and identifying usefull camel V_{HH} fragments, it is critical (i) that camels can be immunized with a variety of antigens, (ii) that the camel V_{HH} genes can be cloned and expressed on filamenteous phages and in E.coli for easy selection with the immobilized antigen by panning, (iii) that the expressed camel V_{HH}'s are properly folded, and (iv) that they have good solubility properties and possess high affinities and specificities towards their antigen.

Camel V_{HH} genes derived from the heavy chain immunoglobins lacking the light chains were previously cloned and analysed (Muyldermans et al., 1994). A comparison of the amino acid sequences of these camel V_{HH} clones clearly showed that the key features for preserving the characteristic immunoglobulin fold are all present. The specific amino acid replacements observed in the camel V_{HH} clones could correlate with the absence of the VL (variable light chains) and the functionality of the camel single V_{HH} domain (Muyldermans et al., 1994).

The invention thus relates to a variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by the following process:

- treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,

- contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3' or 5'-CGCCATCAAGGTACCAGTTGA-3'
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- recovering amplified DNA corresponding to bands of different size orders including:
  . a band of around 750 basepairs which is the amplified product of the variable heavy chain (V_H), CH1, hinge and part of CH2 region of a four-chain immunoglobin,
  . a band of around 620 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,
  . a band of around 550 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,
- purifying the two shortest bands of around 620 and 550 basepairs from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the V_{HH} fragments,
- digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified

fragments when the obtained recombinant vector is used to transform a host cell,

- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
-submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,
- recovering the virions and testing them for their binding activity against the antigen of interest, for example by ELISA,
- recovering the phagemid virions having the appropriate binding activity,
- isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a V_{HH} aminoacid sequence having the appropriate binding activity.

In a preferred embodiment of the invention, the variable V_{HH} fragments are obtainable by adding to the hereabove described amplification step of the cDNA with BACK and FOR primers (p1), a further amplification step with a BACK primer corresponding to the oligonucleotide sequence which has been described hereabove (back p1) and the FOR primer (for p2) having the following nucleotide sequence: 5'- CG ACT AGT

GCG GCC GCG TGA GGA GAC GGT GAC CTG-3'. Not and BstEII sites which can be used for cloning in the pHEN4 vector have been underlined. This FOR primer allows hybridization to the codon position of framework 4 (FR4) region of the V_{HH} nucleotide sequences (amino acid position 113-103).

According to another variant of the process described, this additional amplification step can replace the amplification step which has been described with BACK primer and a FOR primer having respectively the following nucleotide sequences:

5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

5'-CGCCATCAAGGTACCGTTGA-3 ' or

5'-CGCCATCAAGGTACCAGTTGA-3 '

The restriction sites have been underlined.

In another embodiment of the invention the amplification step of the synthetized cDNA is performed with oligonucleotide primers including hereabove described BACK primer and FOR primer having the following sequences:

FOR primer 3: 5'- TGT CTT GGG TTC TGA GGA GAC GGT -3'

FOR primer 4: 5'- TTC ATT CGT TCC TGA GGA GAC GGT -3'

According to this latter embodiment, the V_{HH} fragments of the invention are immediately and specifically amplified by a single amplification (for instance PCR reaction) step when the mixture of FOR primers is used.

These latter primers hybridize with the hinge/framework 4 and short hinge/framework 4 respectively. Each of these FOR primers allows the amplification of one IgG class according to the classification given in patent application WO 94/04678.

The variable V_{HH} fragments corresponding to this definition can also be obtained from other sources of animal cells, providing that these

animals are capable of naturally producing immunoglobulins devoid of light chains according to those described in the previous patent application WO 94/04678.

These variable fragments (V_{HH}) can also be obtained by chemical synthesis or by genetic engineering starting from DNA sequences which can be obtained by the above described process.

The variable fragment of a heavy chain of an immunoglobulin devoid of light chains according to the preceding definitions is specifically directed against an antigen against which the animal has been previously immunized, either by natural contact with this antigen or by administration of this antigen in order to generate an immune response directed against it.

The process which is proposed hereabove to prepare a nucleotide sequence coding for the variable fragments of the invention contains steps of phage display library construction which allow the selection of nucleotide sequences coding for variable fragments of heavy chains having the desired specificity.

According to one preferred embodiment of the invention, the variable fragments of a heavy chain of a immunoglobulin is obtainable from an animal having been previously immunized with a toxin, especially a toxin of a bacteria or a part of this toxin sufficient to enable the production of immunoglobulins directed against this toxin and especially immunoglobulins devoid of light chains.

According to another embodiment of the invention, the variable fragments of a heavy chain of a immunoglobulin is obtainable from an animal having been previously immunized with substances contained in venom of animals.

The antigen used for immunization of the animals is usually under a non toxic form.

The variable fragments according to the invention can be derived from immunoglobulins belonging to different classes especially belonging

to IGg2 or IGg3 immunoglobulin classes, according to the classification given in patent application WO/04678.

In a preferred embodiment of the invention, the variable fragment of a heavy-chain of an immunoglobulin devoid of light chains is directed against the tetanus toxin of Clostridium tetani or against a fragment thereof.

The variable fragments of heavy chains of immunoglobulins devoid of light chains can be also generated against toxins or part thereof from pathogenic organisms such as bacteria and especially can be chosen among the toxins or toxoids of the following bacteria: Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria.

Other antigens appropriate for the preparation of the V_{HH} fragments of the invention can be obtained from the following organism: anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

According to another embodiment of the invention, the variable fragment V_{HH} of a heavy chain of an immunoglobulin devoid of light chains is characterized in that it comprises the following aminoacid sequences:

(Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu

ArgLeuSerCysAla(Ala/Thr)SerGly**(CDR1)**Trp(Phe/Tyr)ArgGlnAlaProGlyLys

Glu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)**(CDR2)**Arg(Phe/Leu)ThrIleSer(Arg/

Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)TyrLeu(Gln/Leu)MetAsnSerLeu

(Lys/Glu)ProGluAspThrAla(Val/Met/Ile)TyrTyrCysAlaAla**(CDR3)**TrpGlyGln

GlyThrGlnValThrValSerSer or

(Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu

ArgLeuSerCysAla(Ala/Tlu)SerGly(Ala,Thr,Ser,Ser/Tyr,Thr,Ile,Gly)(**CDR1**)

Trp(Phe/Tyr)ArgGlnAlaProGlyLysGlu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)

(CDR2) Arg(Phe/Leu)ThrIleSer(Arg/Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)

TyrLeu(Gln/Leu)MetAsnSerLeu(Lys/Glu)ProGluAspThrAla(Val/Met/Ile)Tyr

TyrCysAlaAla**(CDR3)**TrpGlyGlnGlyThrGlnValThrValSerSer,

wherein CDR1, CDR2 and CDR3 represent variable amino acid sequences providing for the recognition of a determined epitope of the antigen used for the immunization of Camelids, CDR1, CDR2 and CDR3 sequences comprising from 5 to 25 amino acid residues preferably CDR1 contains from 7 to 12 amino acid residues, CDR2 contains from 16 to 21 amino acid residues and CDR3 contains from 7 to 25 amino acid residues.

The camel V_{HH} specific amino acid residues Ser 11, Phe 37, Glu 44, Arg 45, Glu 46, Gly 47 are underlined.

One preferred variable fragment according to the invention is encoded by a nucleotide sequence present in recombinant phasmid pHEN4-αTT2(WK6) deposited at the BCCM/LMBP (Belgium) under accession number LMBP3247.

The pHEN4αTT2 (described on Figure 2) is a phasmid carrying a PelB leader signal, a camel V_{HH} gene of which the protein binds tetanus

toxoid, a decapeptide tag (from ImmunoZAP H of Stratacyte) and gene IIIp of M13 in the pUC 119 polylinker between the HindIII and EcoRI sites. This phasmid was transformed in E. coli WK6 cells.

A specific variable fragment according to the invention is for instance characterized in that it comprises the following αTT1 aminoacid sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysAlaAlaSerGlyGlyGlnThrPhe***AspSerTyrAlaMetAla***Trp***Phe***ArgGln

AlaProGlyLysGlu*Cys*GluLeuValSer***Ser***IleIleGly***AspAspAsnArgAsnTyr***

AlaAspSerValLysGlyArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

LeuGlnMetAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln***LeuGly***

SerAlaArgSerAlaMetTyrCysAlaGlyGlnGlyThrGlnValThrValSerSer

According to another preferred embodiment of the present invention, the variable fragment comprises the following αTT2 aminacid sequence :

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

ProGlyLysGlu***Arg***GluGlyValAla***GlyIleSerSerGlyGlySerThrThrAlaTyr***

SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGly***ValSer***

GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

ThrGlnValThrValSerSer

In a preferred embodiment of the invention, the variable V_{HH} fragment of the invention is altered in order to diminish its immunogenic properties. Such a modification can lead to an alternated immunological reaction against the V_{HH} fragments of the invention when they are administered to a host either human or animal, for passive immunoprotection for example.

The invention further relates to a pharmaceutical composition comprising an immunoglobulin heavy chain variable fragment according to those which have been defined hereabove, in admixture with a physiologically acceptable vehicle.

Such pharmaceutical composition can be used for the treatment by passive immunisation, of infections or acute intoxications by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

The present invention further relates to nucleotide sequences coding for a variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, obtainable by the process which has been described hereabove.

Specific nucleotide sequences are those corresponding to αTT1 and αTT2 as described on figures 4A and 4B.

According to an embodiment of the invention, a preferred nucleotide sequence is the sequence contained on phasmid pHEN4-αTT2 deposited at the BCCM/LMBP collection in Belgium on January 31, 1995 under no. LMBP3247.

The invention further provides means for the preparation of bivalent or even multivalent monospecific DNA constructs of variable fragments of an immunoglobulin devoid of light chains and their expression products. It thus gives access to the preparation of monovalent bispecific or multispecific variable constructs obtained from sequences encoding V_{HH} fragments combined with a linker sequence. Bivalent monospecific constructs contain 2 nucleotide sequences coding for V_{HH} fragments directed against the same antigen or epitope. Monovalent bispecific constructs contain on one molecule one nucleotide sequence coding for a V_{HH} fragment directed against one antigen or epitope and another nucleotide sequence coding for a fragment directed against another antigen or epitope.

The corresponding expression products (protein constructs) can be obtained by genetic engeneering especially by expression in host cells, like bacteria (e.g. E. coli) or eukaryotic cells, of the above DNA constructs.

Accordingly a variable fragment of the V_{HH} type having a determined antigen specificity, can be linked to at least one further variable fragment V_{HH} having a determined similar or different specificity in terms of antigen- and/or epitope specificity.

The obtained constructs (in terms of expression products) and especially the bivalent monospecific constructs advantageously offer means to improve the affinity for the antigen(s) against which they are obtained.

The linker sequence between the V_{HH} fragments can be for example a sequence corresponding to the coding sequence of the hinge domain of immunoglobulin devoid of light chains (e.g. the long hinge domain) as

described by (Hamers-Casterman C. et al, 1993) or a sequence derived therefrom.

As an example, in order to ligate these two variable coding sequences of V_{HH} fragments to obtain monovalent bispecific construct, the sequence coding for the hinge and CH_2 domains, especially coding for the long hinge and CH_2 domains of an Immunoglobulin devoid of light chains can be used. These domains have been described in WO 94/04678.

As another example, for instance for the preparation of bispecific or multispecific DNA constructs, the sequence used as linker between the V_{HH} fragments is derived from the coding sequence of the hinge and is devoid of the terminal part containing nucleotides coding for the cysteine residue, or more generally devoid of the codons enabling dimerisation of the V_{HH} fragment.

Preferred linkers include: the sequence starting at nucleotide 400 and ending at nucleotide 479 or between nucleotides 479 and 486 of the nucleotide sequence disclosed on figure 15 or the sequence starting at nucleotide 400 and ending at nucleotide 495 or between nucleotides 487 and 495 of the nucleotide sequence of figure 15.

The linkers can be for instance obtained by digestion of a plasmid containing the coding sequence for the V_{HH}, hinge and CH2 domains of an immunoglobulin devoid of light chains, with Bst EII and XmnI (or KpnI) endonucleases and further amplification of the sequence with primers annealing to each end of the hinge coding sequence as described above and illustrated in the examples.

As an example, constructs (monovalent or multivalent, monospecific or multispecific) can be obtained having a specificity with respect to two or more different toxins or generally antigens of different pathogen organisms including bacteria, viruses...

The invention also relates to a process for the preparation of monovalent bispecific constructs of variable fragments of a heavy chain of an immunoglobulins which comprises the following steps:

a) ligating a nucleotide sequence coding for a variable V_{HH} fragment having a determined antigen- or epitope- specificity to a linker nucleotide sequence to form a V_{HH}-linker fragment;

b) ligating the formed nucleotide sequence coding for the V_{HH}-linker fragment to a nucleotide sequence coding for another V_{HH} fragment having a different antigen- and/or epitope-specificity,

wherein the linker sequence contains the nucleotide sequence coding for part of a hinge domain wherein the codons responsible for the dimerisation of the V_{HH} fragments especially by formation of a disulfide bridge between the last cysteine residues within the hinge domain are deleted.

According to a preferred embodiment, additional steps of ligation are performed with sequences coding for variable fragments (V_{HH} fragments) having the same specificity or a different specificity with respect to the above fragments.

In such a case the V_{HH}-hinge linker - V_{HH} fragment coding sequences recovered from step b) must be digested so as to produce a nucleotide sequence having the following structure hing linker - V_{HH}. In accordance $(V_{HH}$ - hinge linker$)_n$ coding sequences are obtained wherein n is a number higher than 2.

Preferably, the sequence encoding the hinge domain preferably the long hinge domain of the immunoglobulins devoid of light chains is the nucleotide sequence comprising or corresponding to nucleotides 400 to 479 or up to nucleotides 486 of the sequence of figure 15.

In a particular embodiment of the process for the preparation of bivalent or multivalent monospecific or multispecific constructs, the V_{HH} fragment coding sequence linked to a nucleotide sequence encoding the hinge domain has to be amplified. Oligonucleotide primers have been defined which permit the amplification of the sequence of interest. These oligonucleotides anneal respectively with their 3' end to the beginning of

the V_{HH} gene or coding sequence and to the terminal part of the hinge coding sequence. Appropriate primers are for instance:

A4 (Sf I site underlined):

5'CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGA(G,T)GT(G,C)CAGCT-3'

AM007:

5'GGCCATTTGCGGCCGCATTCCATGGGTTCAGGTTTTGG-3'

These chosen primers contain target sequences for specific endonucleases, thus allowing the cloning of the digestion products of the amplified fragments in a suitable vector.

The obtained DNA constructs are then used to transform host cells, for instance E. coli and the expressed proteins are then isolated and purified. The expression products of these DNA constructs are within the scope of the invention.

The heavy-chain antibodies, such as those derived from camel, and their fragments present clear advantages over other antibodies or fragments thereof derived from other animals. These are linked to the distinctive features of the heavy chain antibodies and in particular the novel fragments which can be produced by proteolytic cleavage within the hinge of these heavy-chain antibodies to generate the V_{HH} and the $(V_{HH}h)2$ fragments. The V_{HH} domain of a heavy chain has distinct genetic entities which confer properties of solubility not found in VH fragments derived from conventional antibodies. This property, in addition to its small size and to the fact that the amino acid sequence of the framework region is very homologous to that of human, ensures a minimum of immunogenicity. These properties would allow repetitive treatment with heavy chain V_{HH} fragments for passive immunisation or antibody therapy. As mentioned above, V_{HH} and the $(V_{HH}h)2$ fragments can easily be produced by

proteolytic cleavage of camel immunoglobulins or via recombinant DNA technology.

The most important field of passive immunisation is intoxication due to bacterial toxins and in particular acute intoxication or intoxication due to drug resistant bacteria. Passive immunisation or treatment by antibodies is justified in those cases where vaccination is unpractical or its effects short-lived. They are particularly justified for acute intoxication which if left untreated would have lethal or cripling effects.

The following list of indications is non-exhaustive:

- Tetanos due to infection by Clostridium tetani is an important post-trauma infection and current immunisations are not long lasting. It is also important in the veterinary field.
- Botulism due to ingestion of toxins produced by Clostridium Botulinum and related species.
- Gangrene due to infection by Clostridium.
- Necrotic Enteritis and Enterotoxemia in humans and livestock due to Clostridium Perfringens ingestion.
- Food poisoning due to Staphylococcal endotoxins in those cases where antibiotics are not recommended.
- Pseudomonas infection refractory to antibiotic treatment and in particular ocular infections where rapid intervention is warranted.
- Diphteria toxin infection
- Pasteurella and Yersinia infection causing lethal outcomes in human and livestock.
- Anthrax toxin produced by Bacillus Anthraxis and responsible for one of the five major livestock diseases.
- Infections due to other bacterial agents such as Neisseria or viral agents.

Furthermore, the relative resistance of the V_{HH} fragment to proteolytic cleavage by digestive enzymes (e.g. pepsin, trypsin) offer the possibility of treatment against important gut pathogens, such as Vibrio

cholera and other vibrios, enterotoxic E.Coli, Salmonella species and Shigella or pathogens ingested with food such as Listeria.

Another major target for immunotherapy is in the treatment of intoxication due to bites or contact with toxic invertebrates and vertebrates. Among the invertebrates are sea anemonies, coral and jellyfish, spiders, beas and wasps, scorpions. In the vertebrates, the venemous snakes are of particular importance and in particular those belonging to the families of Viperidae, Crotalidae and lapidea.

Passive immunisation with partially purified immunoglobulins from immunized animals are allready being used. In developing countries, antitetanos and antidiphteria antisera are still produced on a very large scale, usually in horses. Anti-venom antibodies are produced, although on a much smaller scale, against venoms, especially snake venoms.

Another field of application is in combination with the therapeutic use of toxins in medical or surgical practice where neurotoxins such as botulinum toxin are increasingly used.

The invention also relates to the oligonucleotide primers described hereabove, either alone or in kits.

Other characteristics of the invention will appear from the figures and the examples which are described hereafter.

FIGURE 1: 1% agarose gel electrophoresis of the PstI/BstEII digested PCR amplification product of the camel V_{HH} gene (lanes 1 and 2) next to the 123 bp ladder of BRL used as a size marker (lane 4). The PCR product comigrates with the 3rd band of the marker, 369 bp in length.

FIGURE 2: Map of the pHEN4 with the nucleotide sequence of the V_{HH} cloning site shown in the lower part of the figure. The PstI and BstEII sites can be used to clone the camel V_{HH} PCR product shown in Figure 1.

FIGURE 3: 100 individual clones were randomly selected from the original camel V_{HH} library (0), or after the first (1), second (2), third (3) or fourth (4) round of panning. After M13 infection the virions were tested for

binding activity against immobilized tetanus toxoid. The number of positive clones are shown as a function of number of pannings.

FIGURE 4: Nucleotide sequence and the corresponding amino acid sequence of the two identified camel V_{HH} anti tetanus toxoid clones pHEN4-αTT1 and pHEN4-αTT2. The framework SerII, Phe37 and Arg or Cys 45 characteristic for the camel V_{HH} heavy chain antibodies (Muyldermans et al, 1994) are double underlined. The three hypervariable or CDR's according to Kabat et al, (1991) are underlined.

FIGURE 5: SDS-polyacrylamide gel electrophoresis of the proteins extracted from the periplasm of WK6 cultures induced with IPTG. Lane 1 & 8, protein size marker (Pharmacia) MW are (from top of to bottom) 94,000; 67,000; 43,000; 30,000; 20,100 and 14,400 D. Lanes 2 and 7 Expressed periplasmic proteins extracted from WK6 cells containing pHEN4-αTT2' and pHENA-αTT1' cloning vector. Lane 3 & 4, Purified V_{HH} domain of pHEN4-αTT2 at 10 and 1 microgram. Lanes 5 & 6, Purified V_{HH} domain of pHEN4-αTT1 at 10 and 1 migrogram. The position of the expressed soluble camel VH protein is indicated with an arrow. It is clearly absent in the second lane.

FIGURE 6: The total periplasmic extract of 1 liter of culture of WK6 cells carrying the pHEN4-αTT2 was concentrated to 5 ml and fractionated by gel filtration on Superdex 75 (Pharmacia) using 150mM NaCL, 10 mM sodiumphosphate pH7.2 as eluent. The pure V_{HH} is eluted at the fractions between the arrows.

FIGURE 7: CD (Circular dichroism) spectrum (Absorbance versus wavelength in nm) of the purified V_{HH} domain αTT2 at 3.9×10^{-5} Min water measured in a cuvette with a pathlength of 0.2 cm. The negative band near 217 and 180 nm and the positive band around 195 nm are characteristic for β structures (Johnson, 1990).

FIGURE 8: Specificity of antigen binding shown by competitive ELISA. The experiments were carried out in triplicate with the bacterial periplasmic extracts of pHEN4-αTT1 and pHEN4-αTT2.

FIGURE 9: Number of mice surviving after I.P injection of 100 ngr tetanus toxin (10 x LD50) or co-injection of tetanus toxin with the purified V_{HH} αTT1, αTT2 or the non-specific cVH21 (Muyldermans et al., 1994) at 4 or 40 microgram.

FIGURE 10: Variability plot of the camelid V_{HH} sequence (CDR3 and framework 4 regions are not included).

The alignment of the V_{HH} amino acid sequences of camel and lama (a total of 45 sequences) was performed according to Kabat et al. The variability at each position was calculated as the number of different amino acids occuring at a given position, divided by the frequency of the most common amino acid at that position. Positions are numbered according to Kabat et al. The positions above the horizontal bar indicate the amino acids which are referrred to as (CDR1) and (CDR2) in the consensus sequence.

A variability number equal to 1 indicates a perfectly conserved amino acid at that position. The higher the variability number the more likely it will be that the amino acid at this position will deviate from the consensus sequence.

FIGURE 11: Nucleic acid sequence of LYS2 V_{HH} and translation product thereof.

FIGURE 12: Nucleic acid sequence of LYS3 V_{HH} and translation product thereof.

FIGURE 13: Scheme to construct the bivalent monospecific anti-LYS3 camel V_{HH}.

FIGURE 14: Scheme to construct the monovalent bispecific anti-LYS3-long hinge linker-anti-LYS2-Tag.

FIGURE 15: Nucleotide and amino acid sequence of the anti-LYS3-long hinge/Cys-Tag. This protein will spontaneously dimerise.
double underlined: amino acids specific for camellid V_{HH}
Boxed: CDR's
underlined with dashes: long hinge linker
underlined: Tag
Boxed S: Cysteine which is involved in the interdomain disulfide bond.

FIGURE 16: Nucleotide and amino acid sequence of the anti-LYS3-long hinge linker-anti-LYS2-Tag polypeptide.
For underlining and boxes see legend figure 15.

EXAMPLE I: GENERATION OF SPECIFIC CAMEL V_{HH} FRAGMENTS AGAINST TETANUS TOXOID

In this application, results are presented, which prove the feasibility of generating specific camel V_{HH} fragments with demonstrated folding and good binding affinity. This was done by generating a library of camel V_{HH} fragments derived from the dromedary IgG2 and IgG3 isotype, display of the V_{HH} library on phage as fusion proteins with the gene III protein of bacteriophage M13 to allow selection of the antigen binders, and finally of expressing and extracting the soluble and functional V_{HH} fragments from E.coli. As antigen, we choose the tetanus toxoid was chosen because comparisons are possible with published data. In addition, the tetanus toxoid is a highly immunogenic protein that is routinely used as a vaccine in humans to elicit neutralizing antibodies.The two camel V_{HH} fragments that were identified were specific and of high affinity. The affinities of the two camel V_{HH} fragments appear to be comparable with those from the human anti-tetanus toxoid F_{AB}'s recently obtained by Mullinas et al. (1990) and by Persson et al. (1991).

Camel immunization

The serum of a camel (Camelus dromedarius) was shown to be non-reacting with tetanus toxoid (RIT,Smith Kline Beecham, Rixensart, Belgium). This camel was injected with 100 µgr tetanus toxoid at days 9, 30,52, 90 and with 50 µgr at days 220, 293 and 449. The blood was collected 3 days after each injection.

mRNA purification of camel blood lymphocytes

Peripheral blood lymphocytes were purified with Lymphoprep (Nycomed, Pharma) from the bleeding at day 452. Aliquots of 1.10^6 -5.10^6 cells were pelleted and frozen at - 85°C and subsequently used as an enriched source of B-cell mRNA for anti-tetanus toxoid.

The mRNA was prepared from a total of 10^6 peripheral blood lymphocytes either by the "Micro FastTrack" mRNA isolation kit (Invitrogen) or the "QuickPrep Micro mRNA Purification" kit of Pharmacia, following the recommendations of the manufacturer. With both protocols, up to a few µgr of mRNA was obtained which was used in the subsequent cDNA synthesis step.

cDNA synthesis and PCR amplification of camel V_{HH} gene

The first-strand cDNA was synthesized with the Invitrogen "cDNA-cycle" or the Pharmacia "Ready-To-Go"kit. The first-strand cDNA was used immediately afterwards for the specific amplification of the camel V_{HH} region by PCR. The primers used have following sequences : the BACK primer (5'-GA TGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'), the internal PstI site is underlined) is designed to hybridize to the framework 1

region (codons 1 to 10) of the camel V_{HH}, while the FOR primer (5'-CGCCATCAAGGTACCAGTTGA-3') hybridizes in the CH2 region. The PCR was carried out with the Taq polymerase from Boehringer Mannheim.

The PCR product was purified according to standard protocols (Sambrook et al., 1989) and digested with the PstI restriction enzyme of which the target site occurred in the BACK primer, and with BstEII which has a naturally occurring site in the framework 4 of the camel V_{HH} regions. The resulting fragments of approximately 360 bp (FIGURE 1) were ligated into the pHEN4 vector cut with the same restriction enzymes. The pHEN4 vector (FIGURE 2) is the pHEN1 phasmid (Hoogenboom et al., 1991) - a pUC119 based vector -where the myc-tag was replaced by the decapeptide tag present in the ImmunoZAP H vector (Stratacyte). Also the polylinker was modified to allow the cloning of the camel V_{HH} gene between a PstI and a BstEII site located after the PelB leader signal and in front of the decapeptide tag and gene III of bacteriophage M13.

Construction of a camel V_{HH} library

The ligated DNA material was precipitated with 10 volumes and resuspended in 10 µl water and electrotransformed in E.coli XL1 Blue MRF' cells (Stratagene). After electroporation according to the recommended protocol (Stratagene) we kept the cells for 1 hour at 37°C in 1 ml SOC medium before plating on LB plates containing 100 µg ampicilline/ml. After an over night incubation at 37°C the transformed cells were grown out into colonies and some 500,000 recombinant clones were obtained. About 20 colonies, randomly selected, were toothpicked and grown in selective medium (LB/Ampicilline) to prepare plasmid DNA and to check their insert by sequencing. For each clone tested, we found a different V_{HH} region with the aminoacid sequence and contents

characteristic for a V_{HH} originating from a camel heavy chain immunoglobulin (Muyldermans et al., 1994). This indicates that a vast camel V_{HH} library was generated.

The remaining 500,000 clones were scraped from the plates with a minimal amount of LB containing 50% glycerol and stored at -85°C until further use.

Panning with tetanus toxoid

The library was screened for the presence of anti-tetanus toxoid camel V_{HH}'s by panning. To this end, approximately 10^9 cells (=5 ml suspension of the frozen recombinant clones) were grown to midlogarithmic phase in 200 ml of LB medium supplemented with 1% glucose and 100 µg ampicilline/ml before infection with M13K07 bacteriophages. After adsorption of the bacteriophages on the E.coli cells for 30 min at room temperature, the cells were harvested by centrifugation and washed in LB medium supplemented with ampicilline and kanamycin (25µg/ml).The cells were incubated overnight at 37°C to secrete the recombinant pHEN phasmid packaged within the M13virion containing a camel V_{HH} fused to some of its M13gene III proteins (Hoogenboom et al., 1991). The phagemid virions were prepared according to the protocol described by Barbas et al. (1991). The phage pellets were resuspended in blocking solution (1% casein in phosphate buffered saline, PBS), filtered through a 0.2 µm filter into a sterile tube and used for panning. For the panning the Falcon 3046' plates were coated overnight with 0.25 mg/ml or 2 mg/ml tetanus toxoid dissolved in PBS or hydrogencarbonate pH 9.6.The wells were subsequently washed and residual protein binding sites were blocked with blocking solution at room temperature for 2 hours. The adsorption of the phagemid virions on the immobilized antigen and the

washing and elution conditions were according to Marks et al (1991) or were taken from the protocol described by the «Recombinant Phage Antibody System » of Pharmacia 4 consecutive rounds of panning were performed. After the fourth round of panning the eluted phagemid virions were added to exponentially growing TGI cells (Hoogenboom et al. 1991) and plated onampicilline containing LB plates. After overnight growth several colonies were grown individually in LB medium to midlogarithmic growing phase, and infected with M13K07 helper phage. The virions were prepared and tested for their binding activity against tetanus toxoid immobilised on microtiter plates. The presence of the virion binding to the immobilized antigen was revealed by ELISA using a Horse RadishPeroxidase/anti-M13 conjugate (Pharmacia). The percentage of binders was increasing after each round of panning. In the original library we found 3 clones out of 96 which showed binding with the immobilizes tetanus toxoid. This number was increased to 11, 48 and 80 after the first, second and third round of panning. All of the individual clones which were tested after the fourth round of panning were capable to recognize the antigen, as measured by ELISA (FIGURE 3). Ten positive clones were grown and tested by PCR to check the presence of an insert with the proper size of theV_{HH} gene, and their DNA was finally sequenced. The sequencing data revealed that two different clones were present among this set of 10 clones. The phasmid DNA of these clones was named pHEN4-αTT1 and pHEN4-αTT2, (The pHEN4-αTT2 phasmid DNA was deposited at the "BelgianCoordinated Collections of Microorganisms" BCCM/LMBP on January 31, 1995 under accession number LMBP3247), and it was shown that these two different clones contained a cDNA coding for a camel V_{HH} (FIGURE 4). Comparison of the amino acids in these clones with the camel V_{HH} clones analysed before (Muyldermans et al., 1994) clearly indicated that the anti-tetanus camel V_{HH} originated from a heavy chain immunoglobulin lack the CH1 domain and light chains.

Especially the identity of the key residues at position 11 (Ser), 37 (Phe) and 45 (Arg or Cys) and 47 (Leu or Gly) prooved this statement (Muyldermans et al.,1994).

Production of soluble camel V_{HH} with anti-tetanus toxoid activity

The phasmid DNA of the two clones which scored positive in the tetanus toxoid ELISA were transformed into WK6 cells. These cells are unable to suppress the stopcodon present in the vector between the decapeptide tag and the gene III protein. The WK6 *E.coli* cells harboring the pHEN4-aTT1 or pHEN4-aTT2 phasmid were grown at 37°C in 1 liter of TB medium with 100 mgr ampicillin/ml and 0.1% glucose. When the cells reached an OD_{550} of 1.0 we harvested the cells by centrifugation at 5000 rpm,10 minutes. The cell pellet was washed once in TB medium with ampicillin, but ommitting the glucose. The cells were finally resuspended in 1 liter of TB medium with ampicillin (100 µgr/ml). We induced the expression of the camel V_{HH} domain by the addition of 1 mM IPTG and further growth of the cells at 28°C for 16 hours. The expressed proteins were extracted from the periplasmic space following the protocol described by Skerra and Plucthun (1988). We pelleted the *E.coli* cells by centrifugation at 4000g for 10 min. (4°C). The cells were resuspended in 10 ml TES buffer (0.2 M Tris-HCl pH 8.0, 0.5 mMEDTA, 0.5 M sucrose). The suspension was kept on ice for 2 hours. The periplasmic proteins were removed by osmotic shock by addition of 20 ml TES diluted 1/4 with water. The suspension was kept on ice for 1 hour and subsequently centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant contained the expressed camel V_{HH} domain. The extract corresponding to 400 µl cell culture was applied under reducing conditions on a SDS/polyacrylamide protein gel. The extracted proteins were visualized in the SDS/polyacrylamide gels by Coomassie blue staining (FIGURE 5). A protein band with an apparent molecular weight of

16,000 D was clearly present in the E.coli cultures containing the recombinant clones and induced with IPTG. Alternatively, the presence of the camel V_{HH} proteins in the extract was revealed with IPTG. Alternatively,the presence of the camel V_{HH} proteins in the extract was revealed by Western blot using a specific rabbit anti-camel V_{HH} or rabbit anti-dromedary IgG serum or the anti-tag antibody.

We estimate from the band intensity observed in the Coomassie stained gel that more than 10 mg of the camel V_{HH} protein (non-purified) can be extracted from the periplasm of 1 liter induced *E.coli* cells.

For the purification of the anti-tetanus toxoid camel V_{HH} we concentrated the periplasmic extract 10 times by ultrafiltration (*Milipore* membrane with a cut off of 5000 Da). After filtration the concentrated extract from the pHEN4-αTT2 was separated according its molecular weight by gelfiltration on Superdex-75 (*Pharmacia*) (FIGURE 6) equilibrated with PBS (10 mM phosphate buffer pH7.2, 150 mM NaCl). The peak containing the anti-tetanus toxoid activity eluted at the expected molecular weight of 16,000 Da indicating that the protein behaved as a monomer and doesn't dimerize in solution. The fractions containing the pure V_{HH} (as determined by SDS-PAGE) were pooled and the concentration was measured spectrophotometrically using a calculated E_{280} (0.1%) of 1.2 and 2.3 respectively for the αTT1 and αTT2. From the UV absorption at 280 nm of the pooled fraction we could calculate a yield of 6 mgr of purified protein per liter of bacterial culture. The purified protein could be further concentrated by ultrafiltration to 6 mgr/ml in PBS or water without any sign of aggregation, as seen on the UV spectrum.

Concerning the expression yield in *E.coli* it should be realized that at this stage we didn't try to optimize the expression or the protein extraction conditions. However, as the yield of the purified αTT2 camel V_{HH} reached

6 mgr per liter of bacterial culture, and as we obtained the soluble protein at a concentration of 6 mgr/ml, it is clear that the expression is comparable or better than other scFv's or F_{AB}'s expressed in *E.coli*. Furthermore, the solubility of the camel V_{HH} aTT2 is certainly better than that obtained for the mouse VH fragments. The yield and solubility is certainly in the range needed for most applications.

To proove the proper folding of the purified protein, the αTT2 was brought at a concentration of 3.9×10^{-6} M and used it for CD measurement (FIGURE 7). The CD spectrum is characteristic for a polypeptide with a b-pleated sheet folding as expected for a well structurated immunoglobulin fold (Johnson, 1990).

The camel anti-tetanus toxoid V_{HH} affinity measurements

The binding of the camel V_{HH} antibody to the tetanus toxoid immobilised on the microtiter plates was revealed by the successive incubation with firstly, the rabbit anti-camel V_{HH} or rabbit anti-dromedary IgG and secondly a goat anti-rabbit/alkaline phosphatase conjugated antibodies (Sigma). The apparent affinity of the camel V_{HH} proteins against tetanus toxoid was estimated by inhibition ELISA exactly as described by Persson et al. (1991) for the human anti-tetanus toxoid F_{AB} fragments they produced in E.coli

The specificity of the soluble camel V_{HH} for the tetanus toxoid was suggested from the ELISA experiments in which we competed the binding with free antigen was competed. An apparent inhibition constant of around 10^{-7},10^{-8} M was observed for both V_{HH} fragments (FIGURE 8). This compares favorable with the inhibition constants for the human anti-tetanus

toxoid F_{AB} fragments cloned by Persson et al. (1991) which were in the range of $10^{-7}$to 10^{-9} M.

The measurement of the affinity constant by ELISA is however, more reliable if determined according to the procedure of Friguet *et al.* (1987). With this protocol we found an affinity constant of 6.10^7 M^{-1} and 2.10^7 M^{-1} for the αTT1 and αTT2 respectively. These affinities are consistent with a specific V_{HH}-antigen interaction (the polyspecific antibodies generally bind their antigen with affinities of 10^6 M^{-1} or less (Casali *et al.* 1989)).

Epitope recognition of αTT1 and αTT2.

Tetanus toxin consists of three domains. The C fragment binds to the neuronal cells, it is said to be the neurospecific binding domain. The B domain appears to be involved in the neuronal penetration of the A domain or L chain (Montecucco & Schiavo, 1993). The L chain is responsible for the intracellular activity.

The C fragment is the most immunogenic part of the tetanus neurotoxin, and a recombinant C fragment is commercially available (*Boehringer* and *Callbiochem*). We showed by ELISA that the aTT1 bacterial extract binds equally well both to the complete tetanus toxoid and to the recombinant C fragment. Therefore the epitope of this camel V_{HH} is present on the C fragment. By contrast, the αTT2 extract binds to the complete tetanus toxoid, but not to the C fragment. Therefore the αTT2 recognizes an epitope located on the A or B domain.

The *in vivo* neutralization of tetanus toxin toxicity.

The neutralizing activity of the purified camel αTT1 or αTT2 V_{HH} domains against tetanus toxin was tested. As a control, eight NMRI mice of 8 to 12 weeks (80 to 100 gr) were injected I.P. with 400 ngr tetanus toxin

(SmithKline Beecham Biologicals) (= 10 times the LD50) in 0.1 ml PBS. To test the neutralizing activity of the camel V_{HH} αTT1 or αTT2 we preincubated 4 or 40 mgr of this purified recombinant protein with 400 ngr of the tetanus toxin in 0.1 ml of PBS for 30 minutes before I.P. injection into the mice. The survival of the mice was followed over a period of 2 weeks (FIGURE 9). It is clear that all mice injected with the tetanus toxin alone or in the presence of a non-specific purified camel V_{HH} (cVH21 of Muyldermans *et al.*, 1994) were killed within 3 days. The survival of the mice injected with the tetanus toxin was increased significantly by the co-injection of only 4 mgr of the purified camel αTT1 or αTT2. The survival was even more pronounced for the co-injection of tetanus toxin with 40 mgr of camel V_{HH}. It appears that the αTT1 had a sligthly higher neutralizing activity than the αTT2. This could originate from its intrinsic higher affinity for binding the tetanus toxin (Simpson *et al.*, 1990). Alternatively it might result from the binding of the αTT1 V_{HH} to the fragment C of the tetanus toxin which inhibits more the toxic effect than the binding of the αTT2 to its epitope outside the C fragment.

EXAMPLE II : GENERATION OF SPECIFIC CAMEL V_{HH} FRAGMENTS AGAINST LYSOZYME

Using the same protocol as the one described in Example I (specific steps or conditions modifying those of example I are indicated hereafter) for the generation of specific camel V_{HH} fragments having a specificity and an affinity for tetanus toxoid, V_{HH} fragments have been obtained against lysozyme.

We choosed the Hen Egg Lysozyme (HEL) as an antigen to immunize a camel (Camelus dromedarius). This protein was selected for the reason that comparisons can be made with several other mouse

monoclonal antibody fragments recognizing the same antigen and of which the structure even in complex with its antigen are known.

Camel immunization

The serum of a camel was shown to be non-reacting with lysosyme. We injected this camel with 100 µg lysozyme (Boehringer) at days 9, 30, 52, 90 and with 50 µg at days 220, 293 and 449. The blood was collected on average 3 days after each injection.

The following steps were then performed as in Example I.

- mRNA purification of camel blood lymphocytes.
- cDNA synthesis and PCR amplification of camel V_{HH} gene.
- Construction of Camel V_{HH} library.
- Panning with lysozyme (the Falcon 3046' plates were coated with 1 mg/ml lysozyme).

96 colonies were randomly chosen and grown individually in LB medium.

The virions were prepared and tested for their binding activity against lysozyme immobilised on microtiter plates.

The percentage of binders was increasing after each round of panning. Twenty positive clones were grown and tested by PCR to check the presence of an insert with the proper size of the V_{HH} gene, and their DNA was finally sequenced. The sequencing data revealed that two different clones were present among this set of 10 clones. The phasmid DNA of these clones was named pHEN4-αLYS2 and pHEN4-αLYS3, and it was shown that these two different clones contained a cDNA coding for a camel V_{HH} (Figures 11, 12). Comparison of the amino acids in these clones with the camel V_{HH} clones we analysed before (Muyldermans et al., 1994) clearly indicated that the anti-lysozyme camel V_{HH} originated from a heavy chain immunoglobulin lacking the CH1 domain and light chains. Especially

the identity of the key residues at position 11 (Ser), 37 (Phe), 44 (Glu), 45 (Arg) and 47 (Gly) prooved this statement (Muyldermans et al., 1994).

- Production of soluble camel VHH with anti-lysozyme activity.

For the purification of the anti-lysozyme camel V_{HH} we concentrated the periplasmic extract 10 times by ultrafiltration (Milipore membrane with a cut off of 5000 Da). After filtration the concentrated extract from the pHEN4-αLYS2 can be purified by Protein A-Sepharose chromatography. Elution of the αLYS2 V_{HH} is done with 100 mM Tri-ethanol amine. The pH of eluate is immediately neutralized with 1 M Tris-HCl (pH 7.4). Unfortunately the expressed α-LYS3 V_{HH} does not bind to Protein A. Therefore the purification has to be performed by affinity chromatography. The concentrated extract is applied on a colmumn of lysozyme immobilized on CNBr-Sepharose (Pharmacia). Elution of the anti-lysozyme V_{HH} is obtained with 100 mM Tri-ethanolamine. The eluate has to be neutralized as described above. Further purification of both anti-lysozyme V_{HH}'s can obtained by gelfiltration on Superdex-75 (Pharmacia) equilibrated with PBS (10 mM phosphate buffer pH7.2, 150 mM NaCl). The peak containing the anti-lysozyme activity eluted at the expected molecular weight of 16,000 Da indicating that the protein behaved as a monomer and doesn't dimerize in solution. The fractions containing the pure V_{HH} (as determined by SDS-PAGE) were pooled and the concentration was measured spectrophotometrically. A yield of 5 mg of purified protein per liter of bacterial culture was calculated. The purified protein could be further concentrated by ultrafiltration to 10 mg/ml in PBS or water without any sign of aggregation, as seen on the UV spectrum.

- The camel anti-lysozyme V_{HH} affinity measurements

The specificity of the soluble camel V_{HH} for the lysozyme was suggested from the ELISA experiments in which we competed the binding with free antigen. An apparent inhibition constant of around 5.10^{-7} and 5.10^{-8} M was observed for the α-LYS3 and α-LYS2 respectively. These

affinities are consistent with a specific V_{HH}-antigen interaction (the polyspecific antibodies generallly bind their antigen with affinities of 10^6 M^{-1} or less (Casali et al. 1989).

- Epitope recognition of α-LYS2 and α-LYS3.

To analyse whether the two camel V_{HH} with anti-lysozyme activity bind to the same or to different epitopes we used the techniques of additive binding in ELISA (Friguet et al., 1989). An addivity index of more than 40 indicates pairs of antibodies that can bind simultaneously on the antigen, while addivity indices of less than 20 is characteristic for pairs of antibodies with overlapping epitopes. Our camel α-LYS2 and α-LYS3 had an addivity index of 45. From this experiment it appears that the α-LYS2 and α-LYS3 bind to different epitopes on the lysozyme molecule.

EXAMPLE 3 : MAKING BIVALENT MONOSPECIFIC OR MONOVALENT BISPECIFIC BINDING CONSTRUCTS FROM CAMELID V_{HH}'S

From the camel V_{HH}'s with specificity to tetanus toxin (α-TT1 or α-TT2) or with specificity to lysozyme (α-LYS2 or α-LYS3) cloned in the pHEN-4 bacterial expression vector, we made constructs with following characteristics:

1. V_{HH} with ProX repeat sequences of the camel long hinge including the 3 Cys and part of the CH2 domain. These constructs can be also used as an intermediate for the next constructs.

2. V_{HH} with ProX repeat sequences of the hong hinge of camel with one Cys followed by a stopcodon in the pHEN4. These are bivalent constructs with monospecificity.

3. V_{HH} linked with the ProX repeat sequences of the long hinge of camel (without Cys) followed by a second V_{HH}. These are monovalent constructs with bispecificity, or bivalent constructs with monospecificity depending on the V_{HH}'s.

1. ***Camel V_{HH} with camel long hinge and part of CH2 domain.***

The (pHEN4-α-LYS3) or the (pHEN4-α-TT2) plasmids were digested with BstEII and Xmn I. BstEII cuts in the framework 4 of the camel V_{HH}, and Xmn I cuts in the β-lactamase gene of pHEN4. The DNA fragment containing the camel V_{HH} was isolated from agarose gel.

A clone containing a camel V_{HH} with unknown specificity, the camel long hinge and the first part of the CH2 domain cloned in pBluescript (Statagene) was cut with the same enzymes (Bst EII and Xmn I) and the DNA resulting fragment containing the hinge and CH2 parts was isolated from agarose gel.

The two DNA fragments (one containing the V_{HH} of determined specificity, the other containing the coding sequence of the hinge and CH_2 domains) were mixed and ligated to each other and used to transform E. coli cells. As a result a (pHEN4-α-LYS3-long hinge-CH2) plasmid and a (pHEN4-α-TT2-long hinge-CH2) plasmid have been obtained.

2. Bivalent monospecific constructs (Figures 13, 15).

The (pHEN4-α-LYS3-long hinge-CH2) plasmid was taken as template for amplification with primers A4 and AM007.

A4 (Sf I site underlined):

5'CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGA(G,T)GT(G,C)CAGCT-3'

AM007:

5'GGCCATTTGCGGCCGCATTCCATGGGTTCAGGTTTTGG-3'

These primers anneal respectively with their 3' end to the beginning of the V_{HH} and to the end of the structural upper hinge of the camel long hinge sequence. The primer AM007 will extend the 3' end of the α-LYS3 or of the α-TT2 gene (depending on the template) with CCCATGGAATGCGGCCGCAAATGTCC. The NcoI and NotI sites are underlined. These nucleotides up to the Not I site code for the amino acids Pro Met Glu Cys.

The PCR fragment is double digested with Sfi I and Not I, and the resulting fragments are cloned in the pHEN-4 vector cleaved with the same enzymes. The ligated material is transformed in WK6 E. coli cells and selected on ampicillin. The transformed clones are checked for their insert by PCR and by sequencing. The plasmid (pHEN4-α-LYS3-long hinge/Cys) and (pHEN4-α-TT2-long hinge/Cys) were generated.

The extraction of the expressed V_{HH} α-LYS3-long hinge/Cys or α-TT2-long hinge/Cys proteins lead to isolation of a dimerised molecule because of the formation of the disulfide bridge between the Cys residue within the long hinge. Both camel V_{HH} dimer constructs (α-LYS3 long hinge/Cys)$_2$ and α-TT2 long hinge/Cys)$_2$ are well expressed in E. coli upon induction with IPTG, and are easily obtained from the periplasm. They were quite soluble and bound the original antigen with high affinity and high specificity.

3. Monovalent bispecific protein constructs (Figures 14, 16).

In the previous plasmid constructs (pHEN4-α-LYS3-long hinge/Cys) and (pHEN4-α-TT2-long hinge/Cys), we have two restriction sites for Nco I. Digestion of the plasmid with this enzyme allows the isolation of the camel V_{HH} gene followed by the long hinge without the Cys codon. Ligation of the (α-LYS3-long hinge) fragment into the pHEN4-α-LYS2 or in the pHEN4-α-TT2 plasmids linearised with NcoI creates the plasmids (pHEN4-α-LYS3-

long hinge linker-α-LYS2) or (pHEN4-α-LYS3-long hinge linker-α-TT2). Expression of the gene leads to the production of the α-LYS3 V_{HH} linked to the α-LYS2 V_{HH} or linked to the α-TT2 V_{HH} by the intermediate of a linker based on the structural upper hinge of the camel long hinge.

Following this protocol monovalent bispecific proteins consisting of the camel V_{HH} of α-LYS3 linked to the camel V_{HH} of α-LYS2 and the that of camel V_{HH} of α-LYS3 linked to the camel V_{HH} of α-TT2 can be isolated. Both proteins are expressed well in E. coli and can be extracted from the periplasm. In ELISA the binding properties of the latter protein to the tetanus toxoid and to the lysosyme can be shown.

With these gene constructs at hand it becomes possible and straightforward to exchange either V_{HH} with any other V_{HH} with another specificity.

- For example we can exchange the second camel V_{HH} by digesting the plasmid with Pst I, or with Nco I and to ligate the DNA fragment containing the V_{HH}-long hinge linker into the pHEN4-V_{HH} linearised with either Pst I or Nco I.
- Similarly, we exchanged the first camel V_{HH} α-LYS3 gene from the (pHEN4-α-LYS3-long hinge linker-α-LYS2) plasmid construct into (pHEN4-α-TT1-long hinge linker-α-LYS2). This was done by cutting the plasmid with Bst EII and further ligating the DNA fragment containing the (long hinge linker-α-LYS2) into the (pHEN4-α-TT1) plasmid linearised with Bst EII.
- With a slight modification of this protocol it becomes even possible to generate multivalent constructs. In this case the (V_{HH}-long hinge linker-V_{HH}) plasmid needs to be digested with Bst EII and the DNA fragment containing the (long hinge linker-V_{HH}) gene should be isolated from agarose gel. Because of the asymmetry in the recognition site of Bst EII, it is only possible to obtain head-to tail ligations upon self ligation. The

self-ligated DNA should thereafter (with or without prior size selection) be ligated into the pHEN4-V_{HH} plasmid linearised with Bst EII. This will create a plasmid of the type (pHEN4-[V_{HH}-long hinge linker]$_n$).

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CLAIMS

1. Variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by the following process:

- treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,
- contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3 ' or
5'- CGCCATCAAGGTACCAGTTGA-3 '
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- recovering amplified DNA corresponding to bands of different size orders including:

. a band of around 750 basepairs which is the amplified product of the variable heavy chain (V_H), CH1, hinge and part of CH2 region of a four-chain immunoglobin,

. a band of around 620 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,

. a band of around 550 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,

- purifying the two shortest bands from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the V_{HH} fragments,
- digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified fragments when the obtained recombinant vector is used to transform a host cell,
- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
-submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,
- recovering the virions and testing them for their binding activity against the antigen of interest, for example by ELISA,

- recovering the phagemid virions having the appropriate binding activity,
- isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a V_{HH} aminoacid sequence having the appropriate binding activity.

2. Variable fragment (V_{HH}) of a heavy chain of an immunoglobulin, which is encoded by a nucleotide sequence obtainable by a process according to the one disclosed in claim 1, wherein a reamplification step of the 620 basepairs and 550 basepairs PCR product of claim 1 performed with oligonucleotide primers having respectively the following nucleotide sequences:

BACK primer: 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

FOR primer: 5'- CG ACT AGT GCG GCC GCG TGA GGA GAC GGT GAC CTG-3'

3. Variable fragment (V_{HH}) of a heavy chain of an immunoglobulin, which is encoded by a nucleotide sequence obtainable by a process according to the one disclosed in claim 1, wherein the amplification step of the cDNA obtained from the mRNA is performed with oligonucleotide primers having respectively the following nucleotide sequences:

BACK primer: 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

FOR primer 3: 5'- TGT CTT GGG TTC TGA GGA GAC GGT -3'

FOR primer 4: 5'- TTC ATT CGT TCC TGA GGA GAC GGT -3'

4. Variable fragment of a heavy chain of an immuglobulin devoid of light chains according to anyone of claims 1 or 2, encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids wherein the camelids have been immunized with a determined antigen prior to the treatment of their blood lymphocytes or other appropriate cells.

5. Variable fragment of a heavy chain of an immuglobulin according to anyone of claim 1 to 4 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids characterized in that the camelids have been previously immunized with an antigen which is a toxin of a bacteria or the corresponding toxoid.

6. Variable fragment of a heavy chain of an immuglobulin according to claim 5 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids wherein the antigen is the tetanus toxoid of Clostridium tetani.

7. Variable fragment of a heavy chain of an immuglobulin encoded by a nucleotide sequence according to claim 5 wherein the antigen is a bacterial toxin or toxoid chosen among those of the following bacteria: Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria.

8. Variable fragment of a high chain of an immuglobulin according to anyone of claims 1 to 4 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids, wherein the camelids have been immunized with an antigen present in venom of animals.

9. Variable fragment of a high chain of an immuglobulin according to claim 8, encoded by a nucleotide sequence wherein the antigen is a toxin or toxoid chosen among those produced by anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

10. Variable fragment of a heavy chain immunoglobulin encoded by a nucleotide sequence obtained by a process of anyone of claims 1 to 9.

11. Variable fragment of a heavy chain of an immuglobulin, characterized in that it comprises the following aminoacid sequence:

(Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu ArgLeuSerCysAla(Ala/Thr)SerGly**(CDR1)**Trp(Phe /Tyr)ArgGlnAlaProGlyLy s Glu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)**(CDR2)**Arg(Phe/Leu)ThrIleSer(Arg/ Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)TyrLeu(Gln/Leu)MetAsnSerLeu (Lys/Glu)ProGluAspThrAla(Val/Met/Ile)TyrTyrCysAlaAla**(CDR3)**TrpGlyGln GlyThrGlnValThrValSerSer or

(Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu ArgLeuSerCysAla(Ala/Tlu)SerGly(Ala,Thr,Ser,Ser/Tyr,Thr,Ile,Gly)(**CDR1**) Trp(Phe /Tyr)ArgGlnAlaProGlyLysGlu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala) **(CDR2)**Arg(Phe/Leu)ThrIleSer(Arg/Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu) TyrLeu(Gln/Leu)MetAsnSerLeu(Lys/Glu)ProGluAspThrAla(Val/Met/Ile)Tyr TyrCysAlaAla**(CDR3)**TrpGlyGlnGlyThrGlnValThrValSerSer,

wherein CDR1, CDR2 and CDR3 represent variable amino acid sequences providing for the recognition of a determined epitope of the antigen used for the immunization of Camelids, CDR1, CDR2 and CDR3 sequences comprising from 5 to 25 amino acid residues preferably CDR1 contains from 7 to 12 amino acid residues, CDR2

contains from 16 to 21 amino acid residues and CDR3 contains from 7 to 25 amino acid residues.

12. Amino-acid sequence encoded by a nucleotide sequence comprising the sequence starting at nucleotide 400 and ending between nucleotides 479 and 495 of the nucleotides 479 and 495 of the nucleotide sequence presented on figure 15.

13. Variable fragment of a heavy chain of an immuglobulin coded by a nucleotide sequence present in recombinant phasmid pHEN4-αTT2(WK6) deposited at the BCCM/LMBP under accession number LMBP3247.

14. Variable fragment of a heavy chain of an immunoglobulin characterized in that it comprises or it replies to the following αTT1 sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysAlaAlaSerGlyGlyGlnThrPhe***AspSerTyrAlaMetAla***Trp***Phe***ArgGln

AlaProGlyLysGluCysGluLeuValSer***Ser******Ile***IleGly***AspAspAsnArgAsnTyr***

AlaAspSerValLysGlyArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

LeuGlnMetAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln***LeuGly***

SerAlaArgSerAlaMetTyrCysAlaGlyGlnGlyThrGlnValThrValSerSer.

15. Variable fragment of a heavy chain of an immunoglobulin characterized in that it comprises or it replies to the following αTT2 aminoacid sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

ProGlyLysGlu***Arg***GluGlyValAla***GlyIleSerSerGlyGlySerThrThrAlaTyr***

SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGly***ValSer***

GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

ThrGlnValThrValSerSer.

16. Variable fragment of a heavy chain of an immunoglobulin according to anyone of claims 1 to 15 characterized in that it is linked to at least one further variable fragment of heavy chains of an immunoglobulin devoid of light chains according to anyone of claims 1 to 15, the V_{HH} fragments having the same antigen specificity.

17. Bivalent monospecific construct of variable fragments of an immunoglobulin according to claim 16, which is a dimer of V_{HH} fragments having the same specificity.

18. Bivalent monospecific construct according to claim 17, wherein the V_{HH} fragments are linked to each other with the hinge amino-acid sequence of the hinge domain of an immunoglobulin devoid of light chain.

19. Variable fragment of a heavy chains of an immunoglobulin according to anyone of claims 1 to 15 characterized in that it is linked to at least one further variable fragment of heavy chains of an immunoglobulin devoid of light according to anyone of claims 1 to 15, the V_{HH} fragments having different antigen specificities.

20. Multivalent multispecific construct wherein variable fragments according to claim 19 are linked to each other with part of the amino-acid sequence of the hinge domain of an immunoglobulin devoid of light chains,

this part being devoid of at least the codon encoding a cysteine residue at the end of the hinge domain.

21. Multivalent multispecific construct according to claim 20, which contains at least two V_{HH} fragments having a different antigen- and/or epitope- specificity.

22. Construct according to anyone of claims 16 to 18 wherein the sequence of the the hinge domain includes or corresponds to the amino-acid sequence starting at position 400 and ending between position 489 and position 495 as indicated on the sequence of figure 15.

23. Construct according to anyone of claims 19 to 22 wherein the sequence of part of the hinge region includes or corresponds to the amino-acid sequence starting at position 400 and ending between position 479 and position 486 as indicated on the sequence of figure 15.

24. Pharmaceutical composition, characterized in that it comprises an immunoglobulin variable fragment according to anyone of claims 1 to 6 or 19 or a construct according to anyone of claims 17, 18, 20 to 23 in admixture with a physiologically acceptable vehicle and/or adjuvant(s).

25. Pharmaceutical composition according to claim 16 for the treatment by passive immunisation, of infection or acute intoxication by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

26. immunoglobulin variable fragment according to anyone of claims 2 to 6 or 19, or a construct according to anyone of claims 17,18,20 to 23 for use for the treatment by passive immunisation, of infection or acute intoxication by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially

Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

27. Nucleotide sequence coding for a variable fragment (V_{HH}) of a heavy chain of an immunoglobulin obtainable by the following process:

- treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,
- contacting the obtained cDNA with at least two different oligonucleotide primers in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3 ' or
5'-CGCCATCAAGGTACCAGTTGA-3 '
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- recovering amplified DNA corresponding to bands of different size orders including:

. a band of around 750 basepairs which is the amplified product of the variable heavy chain (V_H), CH1, hinge and part of CH2 region of a four-chain immunoglobin,

. a band of around 620 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,

. a band of around 550 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,

- purifying the two shortest bands of 620 and 550 basepairs from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the V_{HH} fragments,
- digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified fragments when the obtained recombinant vector is used to transform a host cell,
- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- harvesting the recombinant host cells, adsorbed with the bacteriophages,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
-submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,

- recovering the virions and testing them for their binding activity against the antigen of interest, for example by ELISA,
- recovering the phagemid virions having the appropriate binding activity,
- isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a V_{HH} aminoacid sequence having the appropriate binding activity.

28. Nucleotide sequence coding for a variable fragment V_{HH} of a heavy chain of an immunoglobulin devoid of light chain, directed against an epitope of the tetanus toxin of Clostridium tetani, characterized in that it codes for an amino acid sequence according to claim 14 or 15.

29. Nucleotide sequence coding for a variable fragment V_{HH} of a heavy chain of an immunoglobulin devoid of light chain, directed against an epitope of the tetanus toxin of Clostridium tetani, characterized in that is comprises one of the following nucleotide sequences:

5

αTT1

```
          10         20         30         40         50         60
          |          |          |          |          |          |
GAGGTGCAGCTGCAGGCGTCTGGGGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGACTC

          70         80         90        100        110        120
          |          |          |          |          |          |
TCCTGTGCGGCCTCTGGGGGACAGACCTTCGATAGTTATGCCATGGCCTGGTTCCGCCAG

         130        140        150        160        170        180
          |          |          |          |          |          |
GCTCCAGGGAAGGAGTGCGAATTGGTCTCGAGTATTATTGGTGATGATAACAGAAACTAT

         190        200        210        220        230        240
          |          |          |          |          |          |
GCCGACTCCGTGAAAGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACGGTATAT

         250        260        270        280        290        300
          |          |          |          |          |          |
CTGCAAATGGACCGTCTGAATCCTGAGGACACGGCCGTGTATTACTGTGCGCAATTGGGT

         310        320        330        340        350
          |          |          |          |          |
AGTGCCCGGTCGGCTATGTACTGTGCGGGCCAGGGGACCCAGGTCACCGTCTCCTCA
```

5

αTT2

```
          10         20         30         40         50         60
GAGGTGCAGCTGCAGGCGTCTGGAGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGGCTC

          70         80         90        100        110        120
TCTTGTACAGCCGCTAATTACGCCTTTGATTCCAAGACCGTGGGCTGGTTCCGCCAGGTT

         130        140        150        160        170        180
CCAGGAAAGGAGCGCGAGGGGGTCGCGGGTATCAGTAGTGGTGGCAGTACCACAGCCTAT

         190        200        210        220        230        240
TCCGACTCCGTGAAGGGCCGATACACCGTCTCCCTTGAGAACGCCAAGAACACTGTGTAT

         250        260        270        280        290        300
CTACTGATAGACAACCTACAACCTGAAGACACTGCCATATACTACTGCGCAGGAGTGAGC

         310        320        330        340        350        360
GGTTGGCGAGGGCGGCAGTGGCTGCTACTGGCAGAGACCTATCGGTTCTGGGGCCAGGGG

         370        380
ACTCAGGTCACCGTCTCCTCA
```

35

30. Nucleotide sequence starting with nucleotide 440 and ending between nucleotide 489 and nucleotide 495 on the nucleotide sequence of figure 15 or this nucleotide sequence in combination with a nucleotide sequence according to anyone of claims 27 or 28.

31. Nucleotide sequence starting with nucleotide 440 and ending between nucleotide 479 and nucleotide 486 on the nucleotide sequence of figure 15 or this nucleotide sequence in combination with a nucleotide sequence according to anyone of claims 27 or 28.

32. Expression product in a host cell of a nucleotide sequence according to anyone of claims 28 to 31.

33. Nucleotide sequence encoding the constructs of anyone of claims 20 to 23.

34. Process for the preparation of monovalent bispecific DNA constructs encoding variable fragments of a heavy chain of an immunoglobulins which comprises the following steps:

a) ligating a nucleotide sequence coding for a variable V_{HH} fragment having a determined antigen- or epitope- specificity to a linker nucleotide sequence to form a V_{HH}-linker fragment;

b) ligating the formed nucleotide sequence coding for the V_{HH}-linker fragment to a nucleotide sequence coding for another V_{HH} fragment having a different antigen- and/or epitope-specificity,

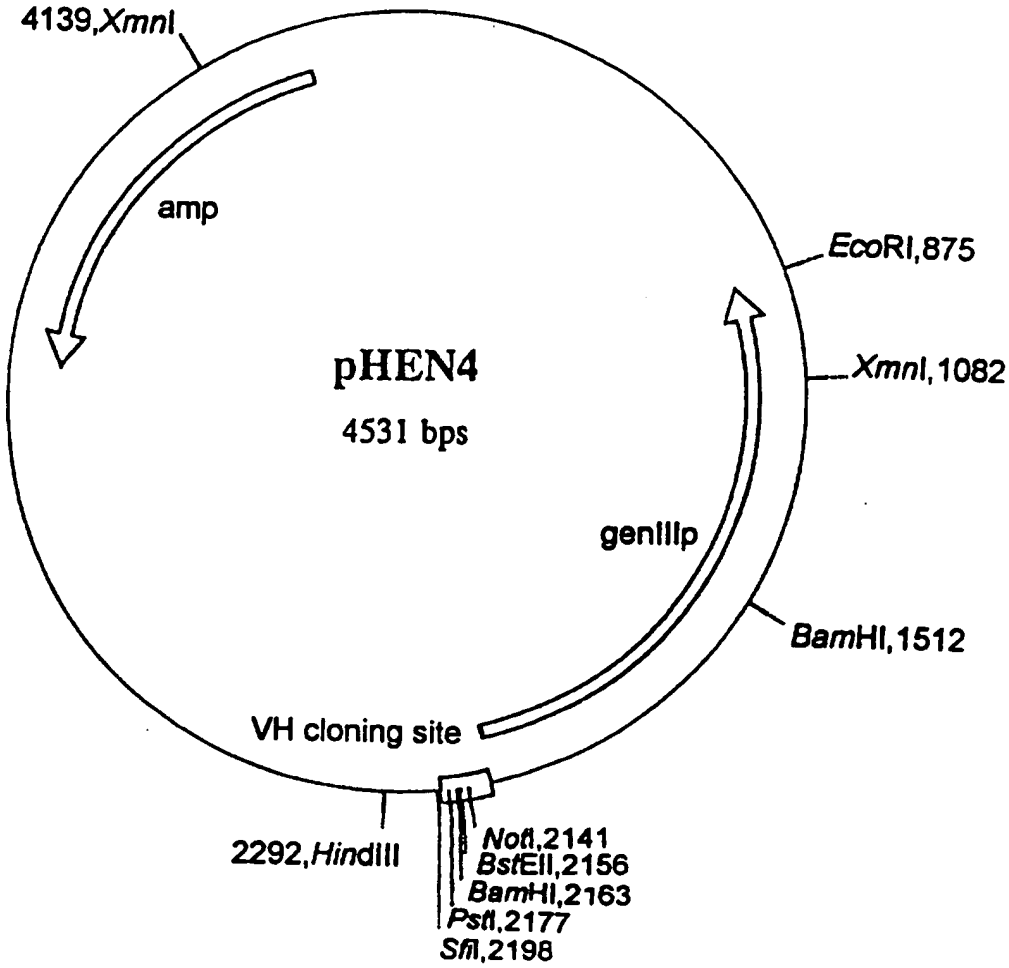
wherein the linker sequence contains the nucleotide sequence coding for part of a hinge domain wherein the codons responsible for the dimerisation of the V_{HH} fragments especially by formation of a disulfide bridge between the last cysteine residues within the hinge domain are deleted.

35. Process according to claim 34 comprising one or several additional step(s) of ligation

36. Process according to claim 34 or 35 wherein the sequence encoding part of the hinge domain comprises or corresponds to the nucleotide sequence starting with nucleotide 400 and ending with one of the

nucleotides between nucleotide 479 and 486 of the nucleotide sequence on the sequence of figure 15.

FIGURE 1



Pel B leader signal - - - I Q V Q L Q *(VH cloning*
GCG GCC CAG CCG GCC ATG GCC CAG CTG CAG CTG CAG GAC CTC
Sfi I Pst I

site) V T V S S I- - - - - - -
GAG GAT CCG GTC ACC GTC TCC AGC GGC CGC TAC CCG TAC GAC
Bst EII Not I

decaptide tag - - - - I I -- *genIIIp*
GTT CCG GAC TAC GGT TCC GGC CGA GCA TAG ACT GTT
Eag I amber

FIGURE 2

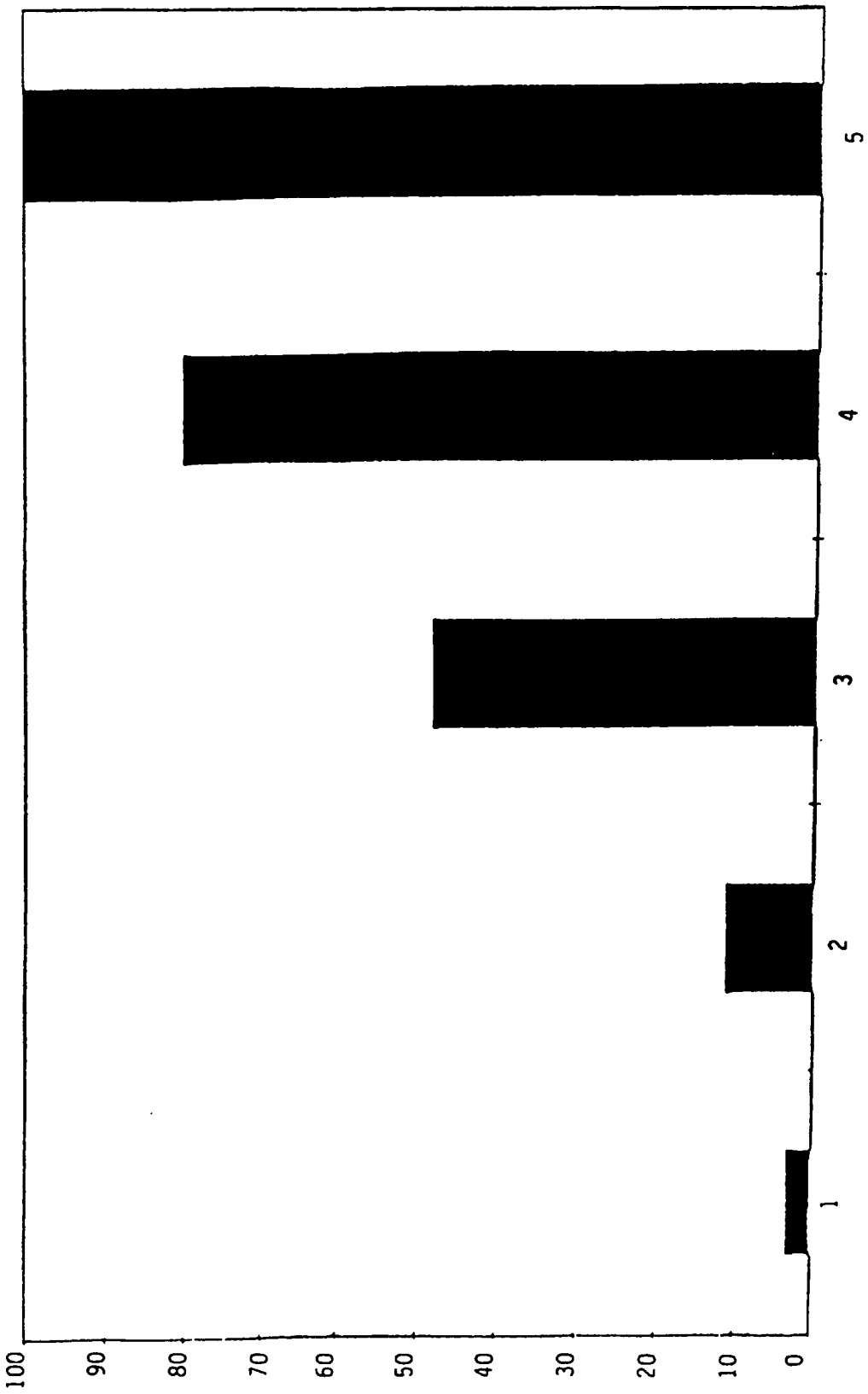


FIGURE 3

pHEN4-αTT1 ----------

```
          10         20         30         40         50         60
          |          |          |          |          |          |
GAGGTGCAGCTGCAGGCGTCTGGGGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGACTC
GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeu

          70         80         90         100        110        120
          |          |          |          |          |          |
TCCTGTGCGGCCTCTGGGGGACAGACCTTCGATAGTTATGCCATGGCCTGGTTCCGCCAG
SerCysAlaAlaSerGlyGlyGlnThrPheAspSerTyrAlaMETAlaTrpPheArgGln

          130        140        150        160        170        180
          |          |          |          |          |          |
GCTCCAGGGAAGGAGTGCGAATTGGTCTCGAGTATTATTGGTGATGATAACAGAAACTAT
AlaProGlyLysGluCysGluLeuValSerSerIleIleGlyAspAspAsnArgAsnTyr

          190        200        210        220        230        240
          |          |          |          |          |          |
GCCGACTCCGTGAAAGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACGGTATAT
AlaAspSerValLysGlyArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

          250        260        270        280        290        300
          |          |          |          |          |          |
CTGCAAATGGACCGTCTGAATCCTGAGGACACGGCCGTGTATTACTGTGCGCAATTGGGT
LeuGlnMETAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGlnLeuGly

          310        320        330        340        350
          |          |          |          |          |
AGTGCCCGGTCGGCTATGTACTGTGCGGGCCAGGGGACCCAGGTCACCGTCTCCTCA
SerAlaArgSerAlaMETTyrCysAlaGlyGlnGlyThrGlnValThrValSerSer
```

FIGURE 4A

pHEN4- αTT2 ----------

```
          10         20         30         40         50         60
          |          |          |          |          |          |
GAGGTGCAGCTGCAGGCGTCTGGAGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGGCTC
GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeu

          70         80         90        100        110        120
          |          |          |          |          |          |
TCTTGTACAGCCGCTAATTACGCCTTTGATTCCAAGACCGTGGGCTGGTTCCGCCAGGTT
SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

         130        140        150        160        170        180
          |          |          |          |          |          |
CCAGGAAAGGAGCGCGAGGGGGTCGCGGGTATCAGTAGTGGTGGCAGTACCACAGCCTAT
ProGlyLysGluArgGluGlyValAlaGlyIleSerSerGlyGlySerThrThrAlaTyr

         190        200        210        220        230        240
          |          |          |          |          |          |
TCCGACTCCGTGAAGGGCCGATACACCGTCTCCCTTGAGAACGCCAAGAACACTGTGTAT
SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

         250        260        270        280        290        300
          |          |          |          |          |          |
CTACTGATAGACAACCTACAACCTGAAGACACTGCCATATACTACTGCGCAGGAGTGAGC
LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGlyValSer

         310        320        330        340        350        360
          |          |          |          |          |          |
GGTTGGCGAGGGCGGCAGTGGCTGCTACTGGCAGAGACCTATCGGTTCTGGGGCCAGGGG
GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

         370        380
          |          |
ACTCAGGTCACCGTCTCCTCA
ThrGlnValThrValSerSer
```

FIGURE 4B

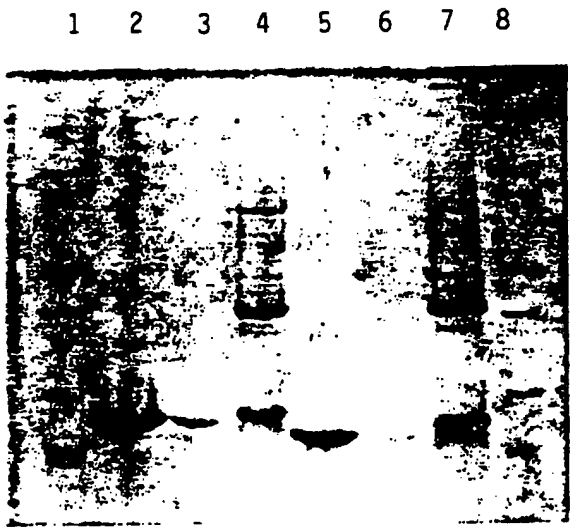


FIGURE 5

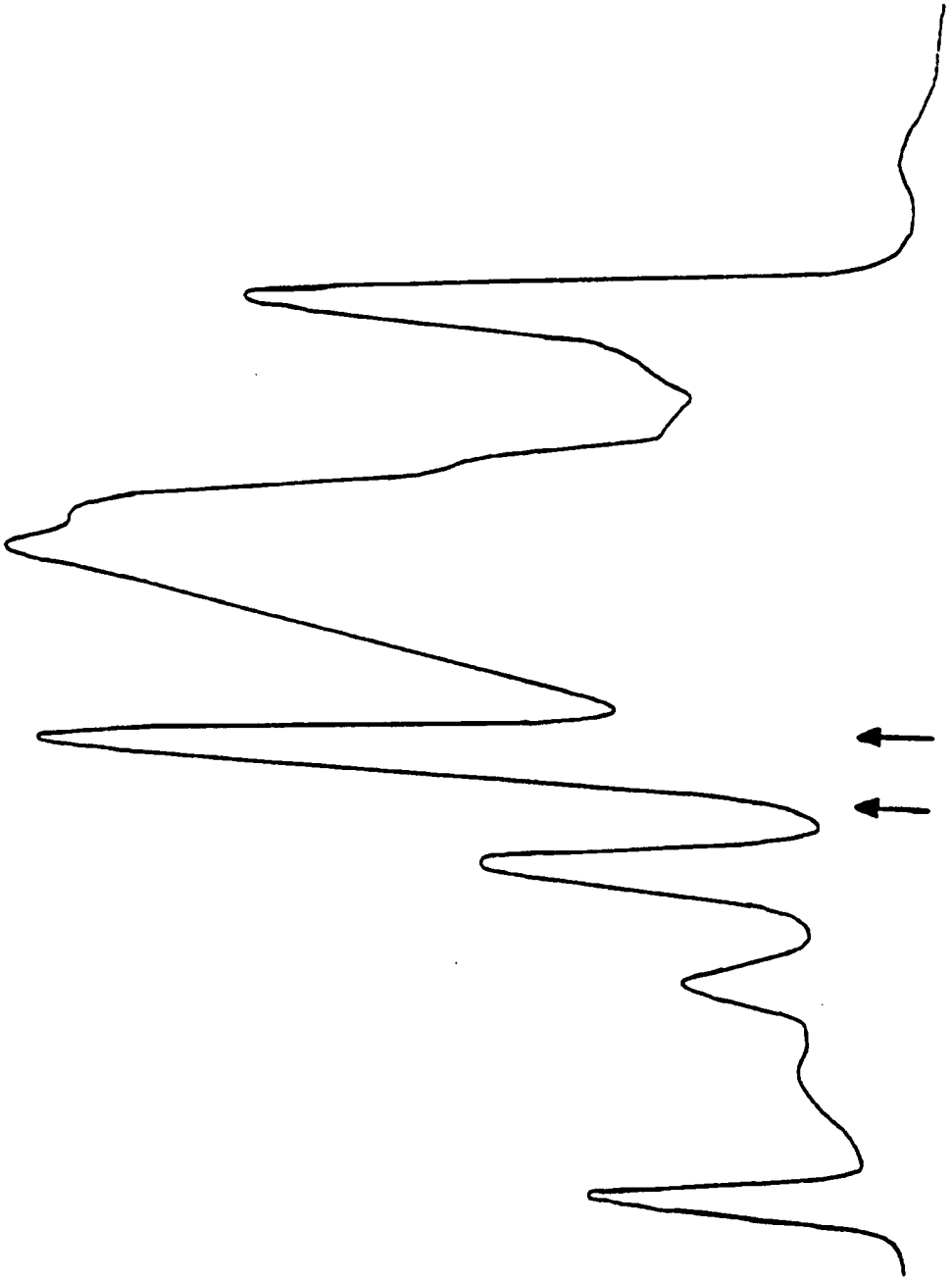


FIGURE 6

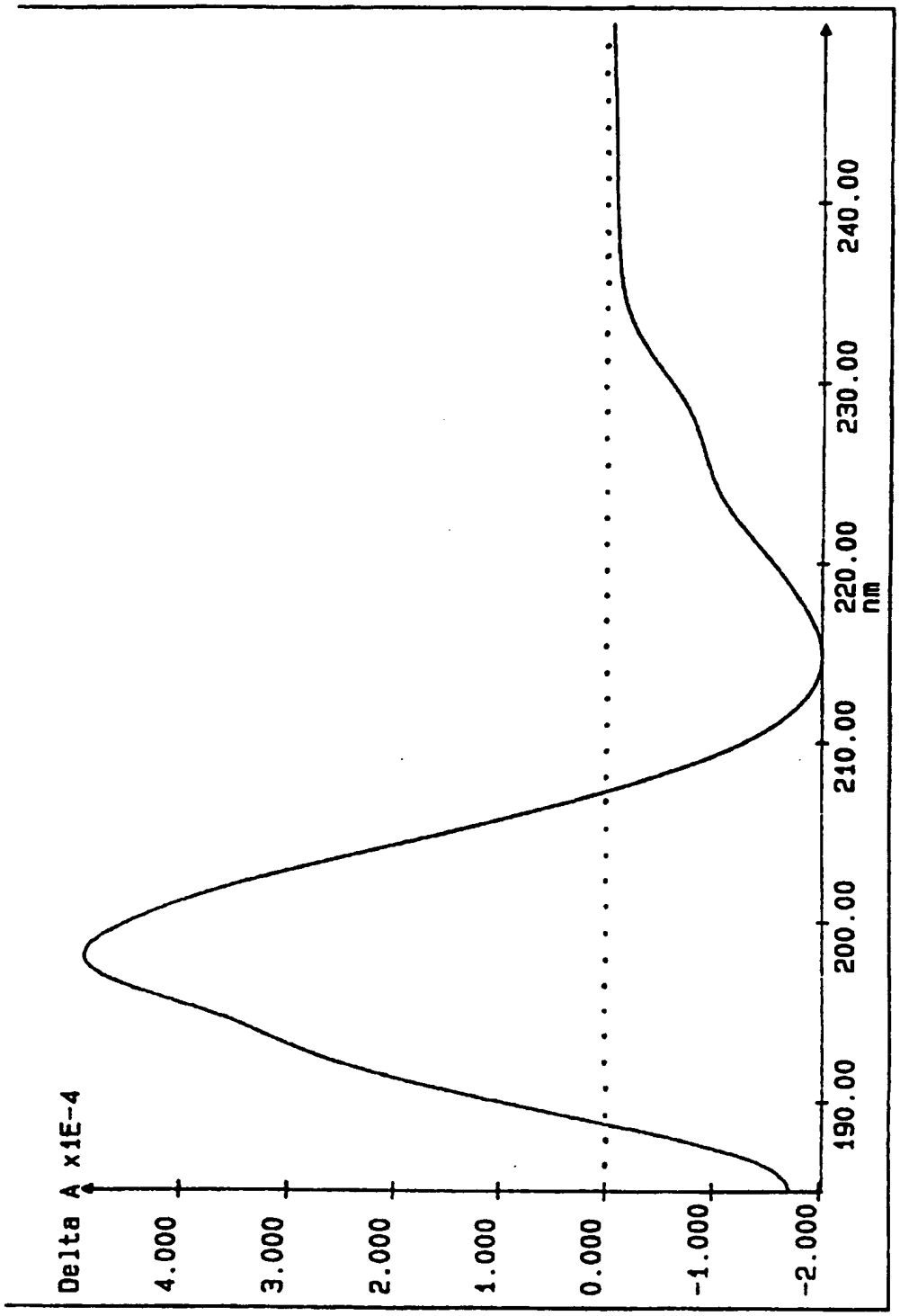

Delta A x1E-4
4.000
3.000
2.000
1.000
0.000
-1.000
-2.000
190.00
200.00
210.00
220.00
230.00
240.00
nm

FIGURE 7

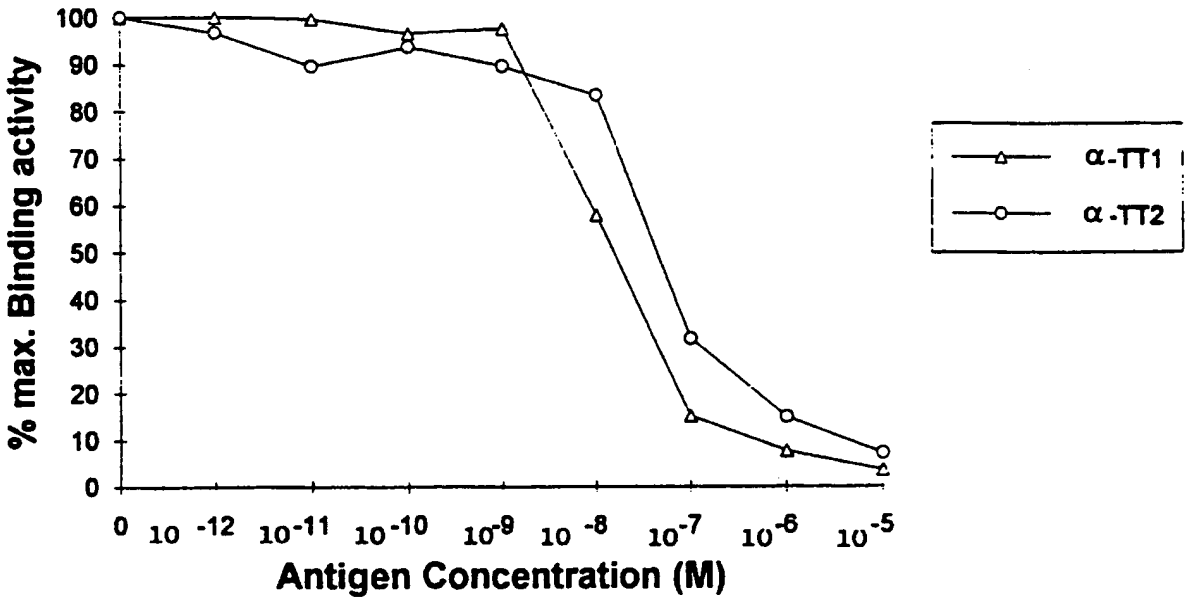

% max. Binding activity
100
90
80
70
60
50
40
30
20
10
0
0 10 -12 10-11 10-10 10-9 10 -8 10-7 10-6 10-5
Antigen Concentration (M)
α-TT1
α-TT2

FIGURE 8

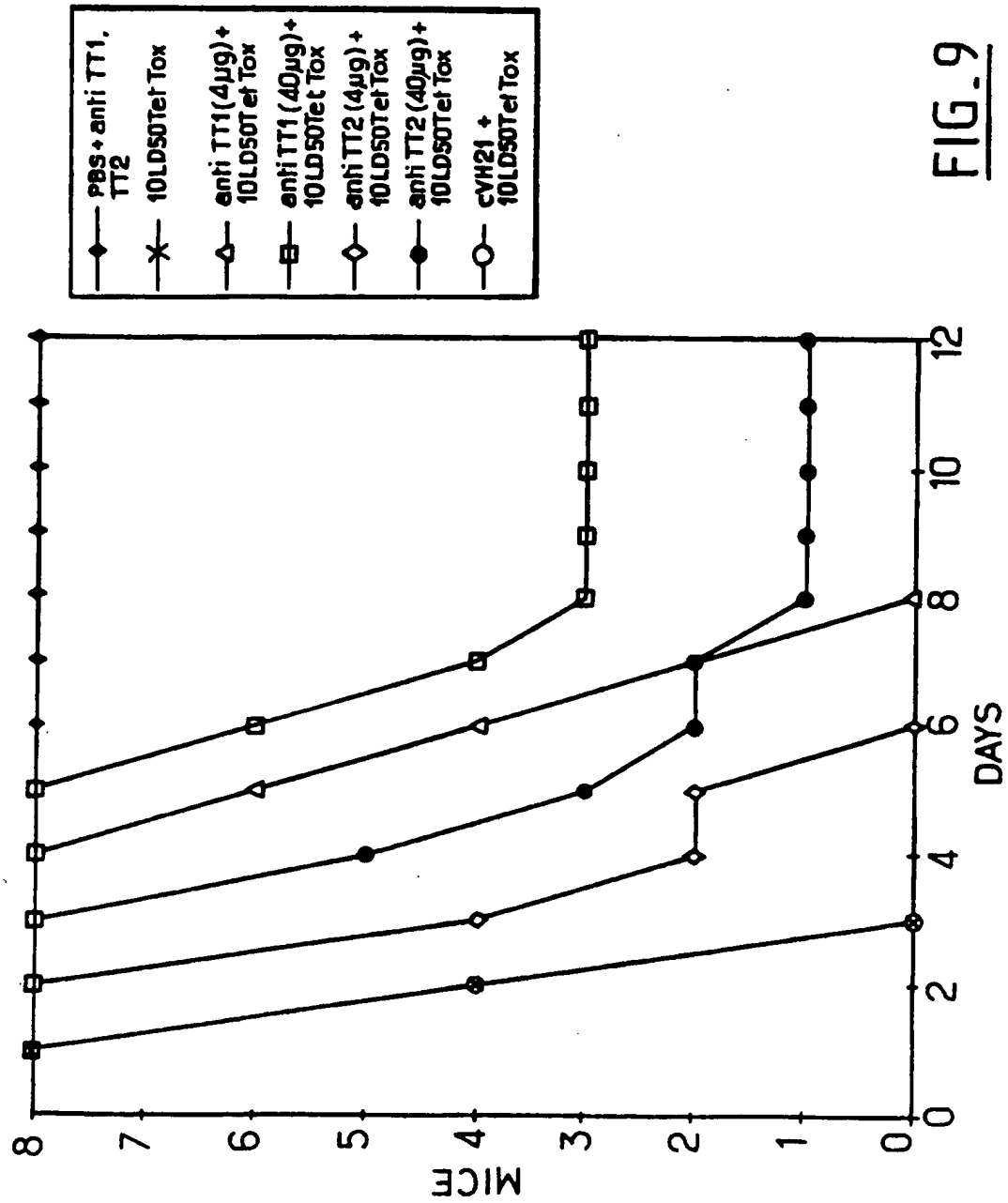

PBS + anti TT1, TT2
10LD50Tet Tox
anti TT1(4µg)+ 10LD50Tet Tox
anti TT1 (40µg)+ 10LD50Tet Tox
anti TT2 (4µg) + 10LD50Tet Tox
anti TT2 (40µg)+ 10LD50Tet Tox
cVH21 + 10LD50Tet Tox
MICE
DAYS
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12

FIG. 9

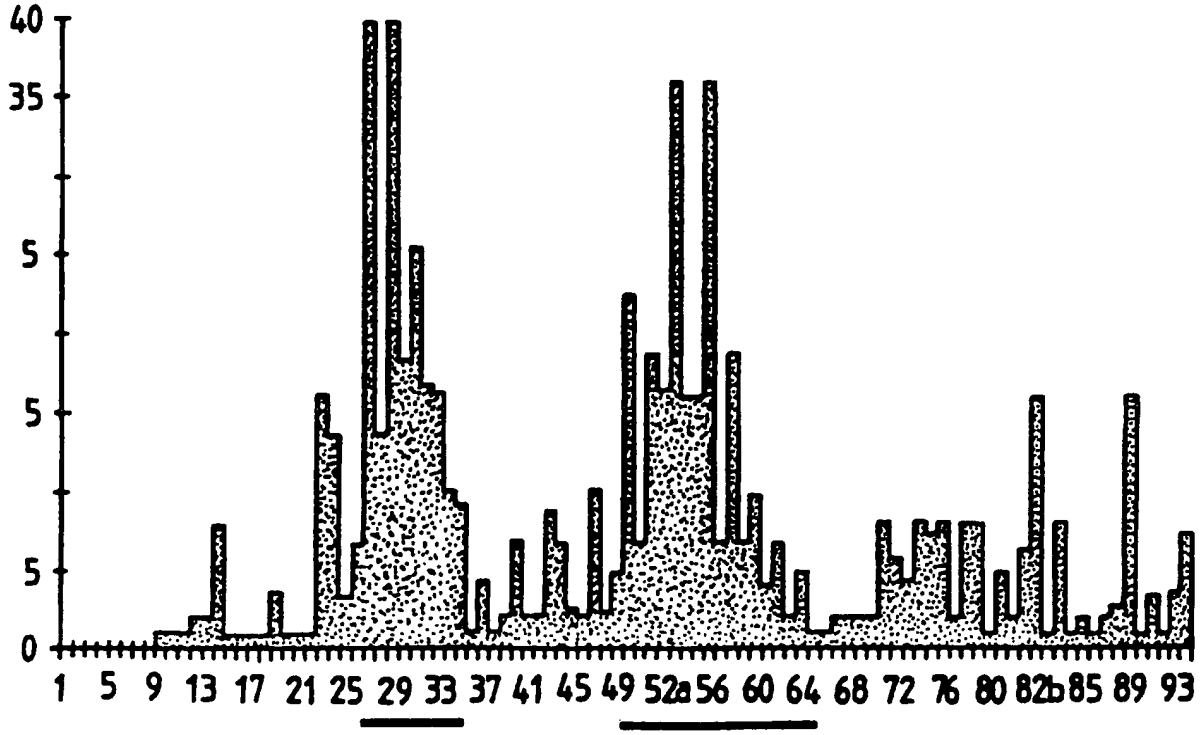

40
35
5
5
5
0
1 5 9 13 17 21 25 29 33 37 41 45 49 52a 56 60 64 68 72 76 80 82b 85 89 93

FIG_10

```
GAG GTC CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA CAG      48
Glu Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gln
                                        11

TCT CTG AGA CTC TCC TGT GCG ACC TCT GGA GCC ACC TCC AGT AGC AAC      96
Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Ala Thr Ser Ser Asn Ser

TGC ATG GGC  TGG TTC CGC CAG GCT CCA GGG AAG GAG CGC GAG GGG GTC    144
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
                 37                          44  45      47
CDR1

GCA GTT ATT GAT ACT GGT AGA GGG AAT ACA GCC TAT GCC GAC TCC GTG    192
Ala Val Ile Asp Thr Gly Arg Gly Asn Thr Ala Tyr Ala Asp Ser Val
                                  CDR2

CAG GGC  CGA TTG ACC ATC TCC TTA GAC AAC GCC AAG AAC ACG CTA TAT   240
Gln Gly Arg Leu Thr Ile Ser Leu Asp Asn Ala Lys Asn Thr Leu Tyr

CTG CAA ATG AAC AGC CTG AAA CCT GAG GAC ACT GCC ATG TAC TAC TGT    288
Leu Gln MET Asn Ser Leu Lys Pro Glu Asp Thr Ala MET Tyr Tyr Cys

GCA GCA GAT ACA TCC ACT TGG TAT CGT GGT TAC TGC GGA ACA AAT CCA    336
Ala Ala Asp Thr Ser Thr Trp Tyr Arg Gly Tyr Cys Gly Thr Asn Pro
                                  CDR3

AAT TAC TTT TCG  TAC TGG GGC CAG GGG ACC CAG GTC ACC GTC TCC TCA   384
Asn Tyr Phe Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
```

FIGURE 11

```
GAT GTG CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA GGG      48
Asp Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
                                        11

TCT CTG AGA CTC TCC TGT GCA GCC TCT GGA TAC ACC ATC GGT CCC TAC      96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ile Gly Pro Tyr

TGT ATG GGG  TGG TTC CGC CAG GCC CCA GGG AAG GAG CGT GAG GGG GTC    144
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
                  37                        44  45      47
CDR1

GCA GCA ATT AAT ATG GGT GGT GGT ATC ACC TAC TAC GCC GAC TCC GTG    192
Ala Ala Ile Asn MET Gly Gly Gly Ile Thr Tyr Tyr Ala Asp Ser Val
                                    CDR2

AAG GGC  CGA TTC ACC ATC TCC CAA GAC AAC GCC AAG AAC ACG GTG TAT   240
Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

CTG CTC ATG AAC AGC CTA GAA CCT GAG GAC ACG GCC ATC TAT TAC TGT    288
Leu Leu MET Asn Ser Leu Glu Pro Glu Asp Thr Ala Ile Tyr Tyr Cys

GCG GCA GAT TCG ACC ATC TAC GCT AGT TAT TAT GAA TGT GGT CAC GGT    336
Ala Ala Asp Ser Thr Ile Tyr Ala Ser Tyr Tyr Glu Cys Gly His Gly
                                    CDR3

CTT TCC ACG GGA GGA TAT GGG TAT GAC TCC  TGG GGC CAG GGG ACC CAG   384
Leu Ser Thr Gly Gly Tyr Gly Tyr Asp Ser Trp Gly Gln Gly Thr Gln

GTC ACC GTC TCC TCA  A                                            400
Val Thr Val Ser Ser
```

FIGURE 12

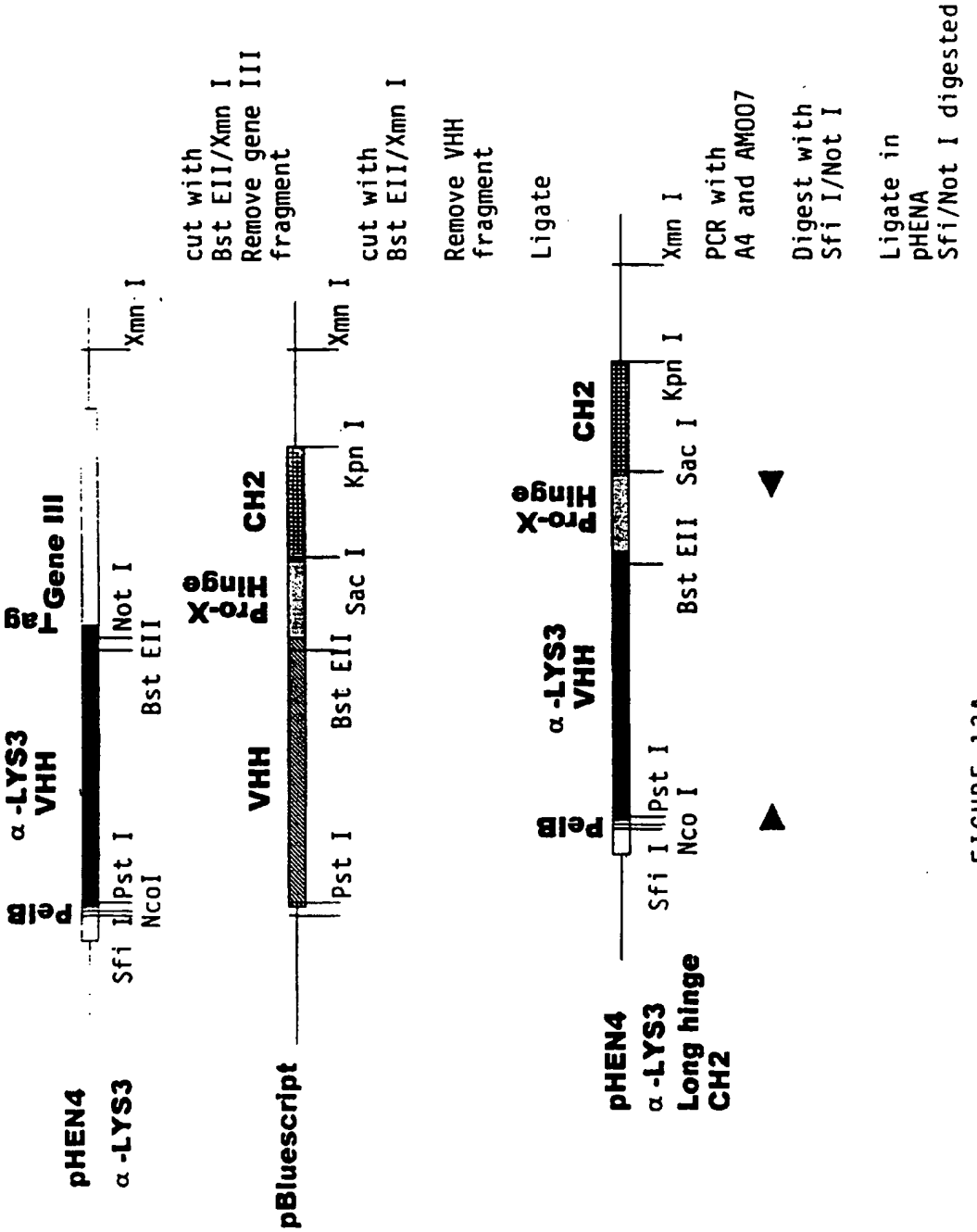

pHEN4
α-LYS3
pelB
α-LYS3
VHH
Sfi I
Pst I
NcoI
Not I
Bst EII
gIII
Gene III
Xmn I
cut with
Bst EII/Xmn I
Remove gene III
fragment
pBluescript
VHH
Pst I
Bst EII
Sac I
Kpn I
Xmn I
Hinge
Pro-X
CH2
cut with
Bst EII/Xmn I
Remove VHH
fragment
Ligate
pHEN4
α-LYS3
Long hinge
CH2
pelB
α-LYS3
VHH
Sfi I
Pst I
Nco I
Bst EII
Sac I
Kpn I
Xmn I
Hinge
Pro-X
CH2
PCR with
A4 and AM007
Digest with
Sfi I/Not I
Ligate in
pHENA
Sfi/Not I digested

FIGURE 13A

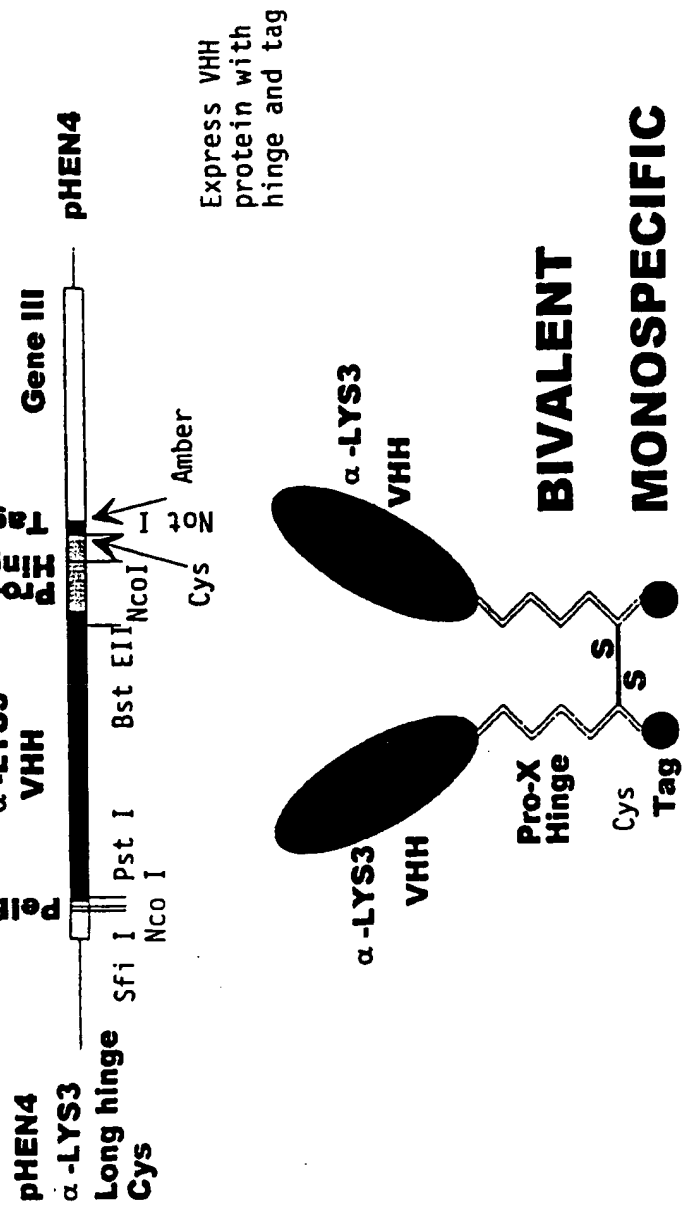

pHEN4
Gene III
Amber
Not I
NcoI
Cys
Pro-X Hinge
Tag
α-LYS3 VHH
Bst EII
Pst I
Nco I
Sfi I
pelB
pHEN4
α-LYS3
Long hinge Cys
Express VHH protein with hinge and tag
α-LYS3 VHH
α-LYS3 VHH
BIVALENT
MONOSPECIFIC
S
S
Pro-X Hinge
Cys
Tag

FIGURE 13B

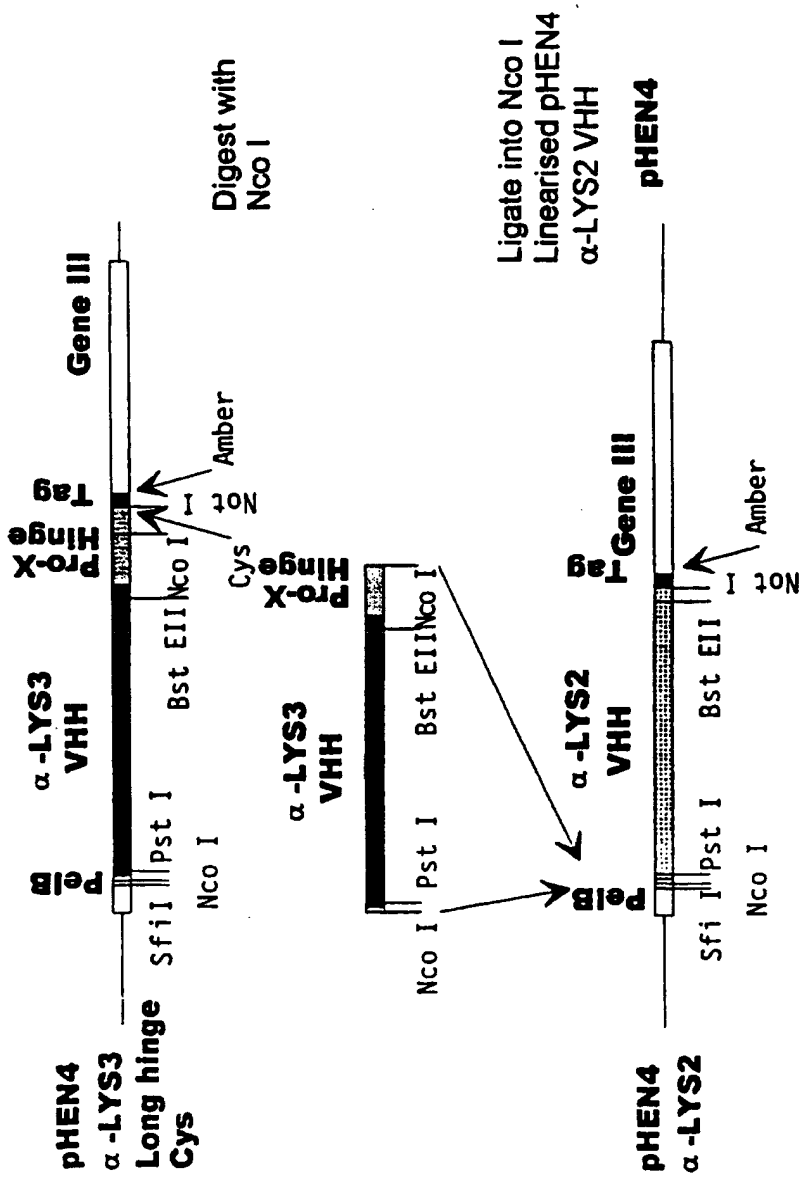

Digest with
Nco I
Ligate into Nco I
Linearised pHEN4
α-LYS2 VHH
pHEN4
Gene III
Amber
Not I
Cys
Pro-X
Hinge
Tag
α-LYS3
VHH
α-LYS2
VHH
Bst EII
Nco I
Pst I
Sfi I
PelB
pHEN4
α-LYS3
Long hinge
Cys
pHEN4
α-LYS2

FIGURE 14A

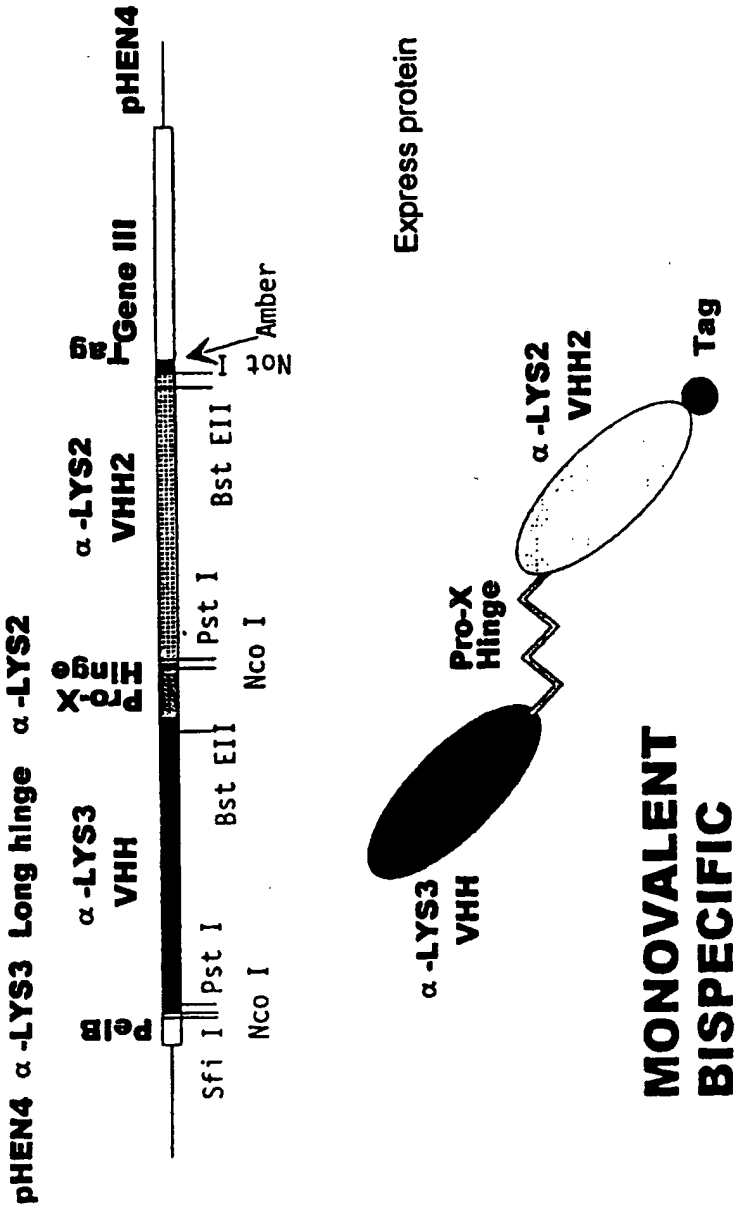

pHEN4 α-LYS3 Long hinge α-LYS2
pelB
α-LYS3 VHH
Pro-X Hinge
α-LYS2 VHH
gene III
pHEN4
Sfi I
Pst I
Nco I
Bst EII
Pst I
Nco I
Bst EII
Not I
Amber
Express protein
α-LYS3 VHH
Pro-X Hinge
α-LYS2 VHH2
Tag
MONOVALENT BISPECIFIC

FIGURE 14B

```
GAT GTG CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA GGG          48
Asp Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
→α LYS3                                  11

TCT CTG AGA CTC TCC TGT GCA GCC TCT GGA TAC ACC ATC GGT CCC TAC          96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ile Gly Pro Tyr

TGT ATG GGG  TGG TTC CGC CAG GCC CCA GGG AAG GAG CGT GAG GGG GTC        144
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
CDR1            37                          44  45      47

GCA GCA ATT AAT ATG GGT GGT GGT ATC ACC TAC TAC GCC GAC TCC GTG         192
Ala Ala Ile Asn MET Gly Gly Gly Ile Thr Tyr Tyr Ala Asp Ser Val
                                CDR2

AAG GGC  CGA TTC ACC ATC TCC CAA GAC AAC GCC AAG AAC ACG GTG TAT        240
Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

CTG CTC ATG AAC AGC CTA GAA CCT GAG GAC ACG GCC ATC TAT TAC TGT         288
Leu Leu MET Asn Ser Leu Glu Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
```

FIGURE 15A

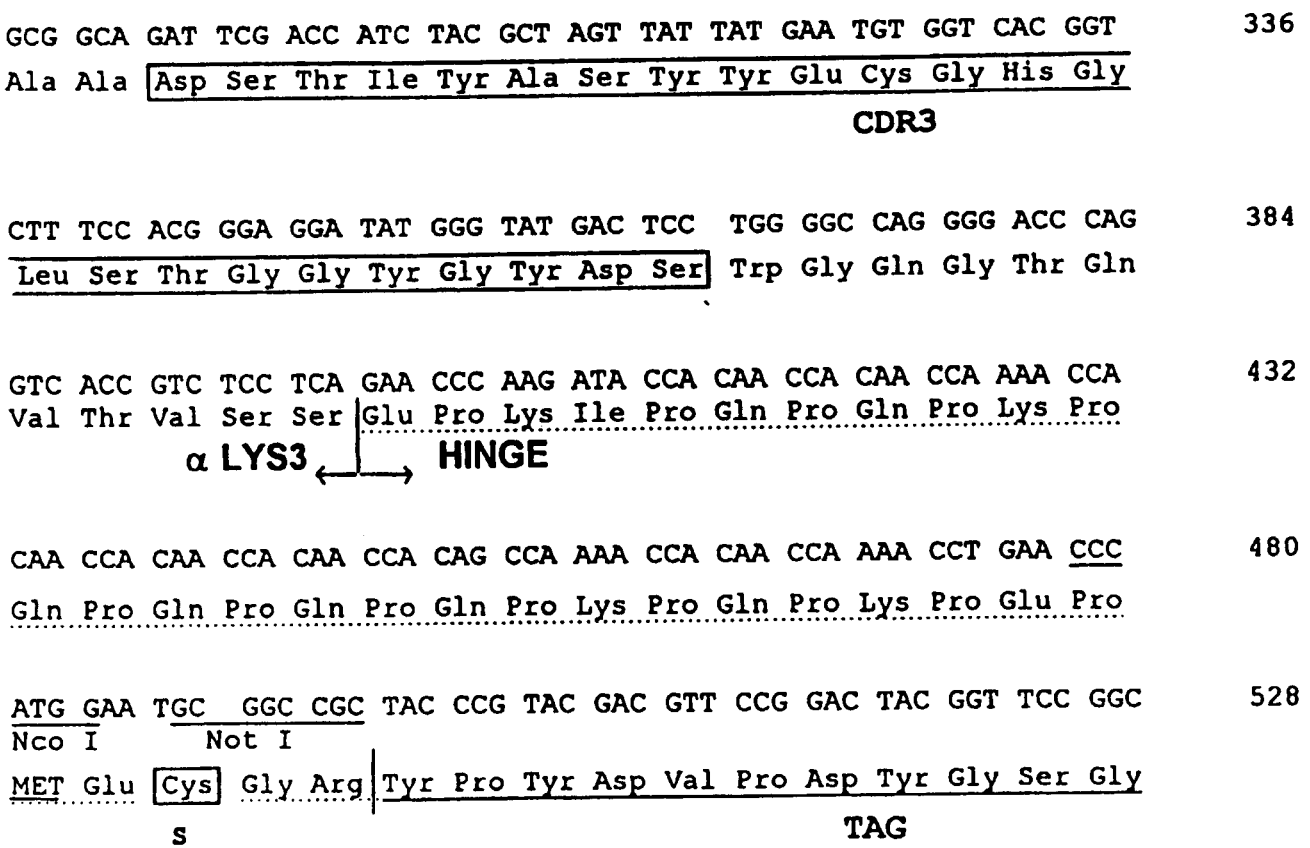

GCG GCA GAT TCG ACC ATC TAC GCT AGT TAT TAT GAA TGT GGT CAC GGT 336
Ala Ala Asp Ser Thr Ile Tyr Ala Ser Tyr Tyr Glu Cys Gly His Gly
CDR3
CTT TCC ACG GGA GGA TAT GGG TAT GAC TCC TGG GGC CAG GGG ACC CAG 384
Leu Ser Thr Gly Gly Tyr Gly Tyr Asp Ser Trp Gly Gln Gly Thr Gln
GTC ACC GTC TCC TCA GAA CCC AAG ATA CCA CAA CCA CAA CCA AAA CCA 432
Val Thr Val Ser Ser Glu Pro Lys Ile Pro Gln Pro Gln Pro Lys Pro
α LYS3
HINGE
CAA CCA CAA CCA CAA CCA CAG CCA AAA CCA CAA CCA AAA CCT GAA CCC 480
Gln Pro Gln Pro Gln Pro Gln Pro Lys Pro Gln Pro Lys Pro Glu Pro
ATG GAA TGC GGC CGC TAC CCG TAC GAC GTT CCG GAC TAC GGT TCC GGC 528
Nco I
Not I
MET Glu Cys Gly Arg Tyr Pro Tyr Asp Val Pro Asp Tyr Gly Ser Gly
S
TAG
CGA GCA TAG 537
Arg Ala ---

FIGURE 15B

```
GAT GTG CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA GGG      48
Asp Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
└→αLYS3                                  11

TCT CTG AGA CTC TCC TGT GCA GCC TCT GGA TAC ACC ATC GGT CCC TAC      96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ile Gly Pro Tyr

TGT ATG GGG TGG TTC CGC CAG GCC CCA GGG AAG GAG CGT GAG GGG GTC     144
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
CDR1            37                          44  45      47

GCA GCA ATT AAT ATG GGT GGT GGT ATC ACC TAC TAC GCC GAC TCC GTG     192
Ala Ala Ile Asn MET Gly Gly Gly Ile Thr Tyr Tyr Ala Asp Ser Val
                          CDR2

AAG GGC  CGA TTC ACC ATC TCC CAA GAC AAC GCC AAG AAC ACG GTG TAT    240
Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

CTG CTC ATG AAC AGC CTA GAA CCT GAG GAC ACG GCC ATC TAT TAC TGT     288
Leu Leu MET Asn Ser Leu Glu Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
```

FIGURE 16A

GCG GCA GAT TCG ACC ATC TAC GCT AGT TAT TAT GAA TGT GGT CAC GGT 336
Ala Ala Asp Ser Thr Ile Tyr Ala Ser Tyr Tyr Glu Cys Gly His Gly
CDR3

CTT TCC ACG GGA GGA TAT GGG TAT GAC TCC TGG GGC CAG GGG ACC CAG 384
Leu Ser Thr Gly Gly Tyr Gly Tyr Asp Ser Trp Gly Gln Gly Thr Gln

GTC ACC GTC TCC TCA GAA CCC AAG ATA CCA CAA CCA CAA CCA AAA CCA 432
Val Thr Val Ser Ser Glu Pro Lys Ile Pro Gln Pro Gln Pro Lys Pro
αLYS3 ← | → HINGE LINKER

CAA CCA CAA CCA CAA CCA CAG CCA AAA CCA CAA CCA AAA CCT GAA CCC 480
Gln Pro Gln Pro Gln Pro Gln Pro Lys Pro Gln Pro Lys Pro Glu Pro
LINKER HINGE ←

ATG GCA GAG GTC CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT 528
Nco I
MET Ala Glu Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala
→ αLYS2 11

GGA CAG TCT CTG AGA CTC TCC TGT GCG ACC TCT GGA GCC ACC TCC AGT 576
Gly Gln Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Ala Thr Ser Ser

FIGURE 15B

```
AGC AAC TGC ATG GGC  TGG TTC CGC CAG GCT CCA GGG AAG GAG CGC GAG      624
Ser Asn Cys MET Gly  Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu
    CDR1                 37                          44  45

GGG GTC GCA GTT ATT GAT ACT GGT AGA GGG AAT ACA GCC TAT GCC GAC      672
Gly Val Ala Val Ile Asp Thr Gly Arg Gly Asn Thr Ala Tyr Ala Asp
47                                  CDR2'

TCC GTG CAG GGC  CGA TTG ACC ATC TCC TTA GAC AAC GCC AAG AAC ACG     720
Ser Val Gln Gly  Arg Leu Thr Ile Ser Leu Asp Asn Ala Lys Asn Thr

CTA TAT CTG CAA ATG AAC AGC CTG AAA CCT GAG GAC ACT GCC ATG TAC      768
Leu Tyr Leu Gln MET Asn Ser Leu Lys Pro Glu Asp Thr Ala MET Tyr

TAC TGT GCA GCA GAT ACA TCC ACT TGG TAT CGT GGT TAC TGC GGA ACA      816
Tyr Cys Ala Ala Asp Thr Ser Thr Trp Tyr Arg Gly Tyr Cys Gly Thr
                                    CDR3

AAT CCA AAT TAC TTT TCG TAC  TGG GGC CAG GGG ACC CAG GTC ACC GTC     864
Asn Pro Asn Tyr Phe Ser Tyr  Trp Gly Gln Gly Thr Gln Val Thr Val

TCC AGC GGC CGC | TAC GAC GTT CCG GAC TAC GGT TCC GGC CGA GCA TAG    912
      Not I
Ser Ser | Gly Arg | Tyr Asp Val Pro Asp Tyr Gly Ser Gly Arg Ala ---
αLYS2↵                                  TAG
```

FIGURE 16C

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC:/EP 96/01725

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 04678 (C. CASTERMAN ET AL.) 3 March 1994 cited in the application see claims ---	1-36
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 87, no. 20, WASHINGTON, DC, USA, pages 8095-8099, XP002011332 R. MULLINAX ET AL.: "Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library." cited in the application see the whole document --- -/--	1-6, 24-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 1996	0 3. 09. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROTEIN ENGINEERING, vol. 7, no. 9, OXFORD, GB, pages 1129-1135, XP002011333 S. MUYLDERMANS ET AL.: "Sequence and structure of Vh domain from naturally occurring camel heavy chain immunoglobulins lacking light chains." cited in the application see abstract see figures	11
A	FEBS LETTERS, vol. 339, no. 3, 21 February 1994, AMSTERDAM, NL, pages 285-290, XP002011334 J. DAVIES ET AL.: "'Camelising' human antibody fragments: NMR studies on VH domains." see the whole document	11
A	WO,A,93 01288 (DEUTSCHES KERNFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 21 January 1993 see the whole document	1,27
A	WO,A,94 25591 (UNILEVER N.V.) 10 November 1994 see examples see claims	1-15, 24-32
A	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 50, no. 8, CHICAGO, IL, USA, pages 1279-1281, XP002011335 J. PAUL-MURPHY ET AL.: "Immune response of the llama (Lama glama) to tetanus toxoid vaccination." see abstract	4-6,28
P,A	BIO/TECHNOLOGY, vol. 13, no. 5, USA, pages 475-479, XP002011336 J. DAVIES ET AL.: "Antibody VH domains as small recognition units." see the whole document	1-15, 24-32

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PC1/EP 96/01725

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9404678	03-03-94	EP-A-	0584421	02-03-94
		AU-B-	4949793	15-03-94
		CA-A-	2142331	03-03-94
		EP-A-	0656946	14-06-95
		FI-A-	950782	20-04-95
		JP-T-	8500487	23-01-96
		ZA-A-	9306086	22-07-94
WO-A-9301288	21-01-93	DE-A-	4122599	04-02-93
		EP-A-	0547201	23-06-93
		JP-T-	6500930	27-01-94
WO-A-9425591	10-11-94	AU-B-	6796094	21-11-94
		EP-A-	0698097	28-02-96